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COST EFFECTIVE HUMAN PROTEIN C PURIFICATION  
FROM COHN FRACTION IV-1 USING MINI-ANTIBODY

By

Doh Gyeuhn Ahn

B.S., Kyung Hee University, Republic of Korea, 1995

M.S., Kyung Hee University, Republic of Korea, 1997

A Dissertation

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Graduate School of the University of Louisville  
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for the Degree of

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Department of Chemical Engineering  
University of Louisville  
Louisville, Kentucky

August 2005

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## **DEDICATION**

This dissertation is dedicated to my mother

Ms. Chang Woon Kim,

who has given me invaluable educational opportunities and prayed for me.

## ACKNOWLEDGMENTS

I would like to acknowledge the American Red Cross for the supply of PC and Dr. Michael Sierks for providing the mini-Mab producing *E. coli* colonies. I would like to thank my major professor, Dr. Kyung A. Kang for her guidance. I would like to thank the other committee members, Drs. Duane F. Bruley, Thomas L. Starr, James C. Watters, Muriel C. Maurer, and Frank P. Zamborini, for their valuable comments and suggestion. Many thanks are given to the faculty, staff, and students of Chemical Engineering Department and Speed School of Engineering who have given me helpful assistance and encouragement. I would also like to express my special thanks to my wife, Hyun Sun Youn, and my daughter, Ha Eun Ahn for their patience, love, motivation, and encouragement.

## ABSTRACT

### COST EFFECTIVE HUMAN PROTEIN C PURIFICATION FROM COHN FRACTION IV-1 USING MINI-ANTIBODY

Doh Gyeuhn Ahn

August 5, 2005

Protein C (PC) is an important anticoagulant, antithrombotic, and anti-inflammatory in blood plasma. PC deficiency can lead to severe venous thrombotic events, including lung embolism, stroke, and heart attack. In the body, PC is activated only when it is needed, and, therefore, PC does not cause bleeding problems that currently available anticoagulants may do.

Purification of PC from plasma is currently done by immunoaffinity chromatography using monoclonal antibodies (Mabs), which is very expensive. Single chain variable regions (mini-Mab) with a PC binding capability, which can be produced in recombinant *E. coli*, were developed for PC purification. Compared to Mabs, mini-Mabs are easier to control the contamination during the production, with lower production cost, and easier to scale-up the process. In this Ph.D. research, the effect of media conditions on the PC mini-Mab production, the mini-Mab purification yield from the production media, and the PC purification performance of the mini-Mab from Cohn Fraction IV-1 (an inexpensive PC source) were studied.

The optimum conditions for mini-Mab production medium were determined to be 0.1 % glucose, 0.1 mM IPTG, initial pH 5.5~6.0, 23~30 °C of media temperature during

the production, and 18 hours of mini-Mab production time. The final mini-Mab production level became 450 mg/L.

Mini-Mab in the production medium broth was purified using protein A affinity chromatography. A purification yield of 34 % was obtained using the elution pH of 3.0.

For PC purification using the PC mini-Mab, among four commercially available affinity chromatography matrices, NHS Sepharose showed the best performance. The PC mini-Mab immobilized NHS Sepharose, when Cohn Fraction IV-1 is used as the source material, showed a PC purification yield of 16 %. High human serum albumin (HSA) content in the source material was found to reduce PC purification yield significantly. Therefore, anion exchange chromatography (DEAE) was used for the pretreatment of the Cohn Fraction IV-1 before the affinity chromatography. DEAE chromatography removed over 99 % of the human serum albumin from Cohn Fraction IV-1. The PC purification yield from the eluate of DEAE chromatography of Cohn Fraction IV-1 was approximately 25 %, increasing by approximately 10 %. PC production cost using the mini-Mab immobilized NHS-Sepharose matrix was estimated to be approximately 100 times cheaper than that using regular Mab.

## TABLE OF CONTENTS

	PAGE
ACKNOWLEDGMENTS .....	iv
ABSTRACT .....	v
LIST of TABLES .....	ix
LIST of FIGURES .....	xi
I. INTRODUCTION .....	1
II. BACKGROUND .....	3
A. Protein C .....	3
B. PC Sources .....	5
C. Methods Used for the Purification of PC from Plasma .....	7
D. PC Homologues .....	9
E. Mini-Antibody Specific against PC .....	10
F. Previous Study on PC Purification Using Mini-Mab .....	12
III. MATERIALS, INSTRUMENTS, AND METHODS .....	14
A. Materials .....	14
B. Instruments .....	17
C. Methods .....	18
IV. RESULTS AND DISCUSSION .....	29



A. Process Optimization of the Mini-Mab Production .....	29
B. Mini-Mab Purification .....	43
C. PC Purification .....	45
D. Economic Analysis of PC Purification by Mini-Mab .....	61
V. CONCLUSIONS .....	64
VI. FUTURE WORK .....	66
REFERENCES .....	67
NOMENCLATURE .....	78
CURRICULUM VITAE .....	81

## LIST OF TABLES

TABLE	PAGE
1. Properties of vitamin K-dependent proteins .....	10
2. The effect of a membrane reactive cationic peptide (phospholipase A2) and a detergent (Triton X-100) on the mini-Mab release from the cell .....	40
3. The effect of PMSF on the mini-Mab production .....	41
4. The effect of PMSF on the mini-Mab storage .....	42
5. Mini-Mab purification by Protein A Sepharose <sup>TM</sup> .....	43
6. PC purification performance by mini-Mab immobilized matrices .....	48
7. PC purification efficiency when 2 mg, 4 mg, or 6 mg of the mini-Mab was immobilized in 1 ml of the matrix. ....	50
8. Amounts of PC and PC homologues in Cohn Fraction IV-1 paste .....	52
9. Amount of PC and PC homologues in various fractions during purification process from Cohn Fraction IV-1 at pHs of 6.0 and 7.0 .....	53
10. Effect of HSA on PC purification .....	55
11. Effect of the salt concentration in washing buffer on the purification of PC from Cohn Fraction IV-1 .....	56
12. The amount of PC, PC homologues, and HSA in the DEAE eluate of Cohn Fraction IV-1 .....	59
13. PC purification from the DEAE eluate of Cohn Fraction IV-1 using mini-Mab Immobilized NHS matrix .....	60
14. An economic analysis of the production for the mini-Mab from <i>E. coli</i> and for the Mab from animal cell culture .....	62

15. Economic analysis of PC purification using mini-Mab and regular Mab .....	63
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## LIST OF FIGURES

FIGURE	PAGE
1. Schematic diagram of Cohn Fractionation .....	7
2. Structure of Mab .....	11
3. Standard curve for the purified mini-Mab concentration .....	26
4. Mini-Mab production levels by thirteen <i>E. coli</i> colonies .....	30
5. The effect of glucose concentration on mini-Mab production .....	31
6. Cell growth curve of <i>E. coli</i> HB2151 E4FX .....	32
7. Mini-Mab production time after adding of IPTG .....	33
8. The effect of initial pH of the production media on the mini-Mab production .....	34
9. The effect of temperature on mini-Mab production .....	35
10. The effect of IPTG concentration on mini-Mab production .....	36
11. The effect of lactose as an inducer on mini-Mab production .....	37
12. The effect of sucrose on the mini-Mab release .....	39
13. Electrophoresis of the samples of the mini-Mab purification from the production supernatant on Protein A Sepharose <sup>TM</sup> .....	44
14. Cross reactivity of mini-Mab against PC and PC homologues .....	45
15. Electrophoresis of the samples from PC purification from Cohn Fraction IV-1 using the mini-Mab immobilized NHS Sepharose <sup>TM</sup> .....	57
16. Electrophoresis of the eluate of PC purification using the mini-Mab immobilized NHS Sepharose <sup>TM</sup> with the source of the DEAE eluate .....	61

## **CHAPTER I**

### **INTRODUCTION**

Protein C (PC) is a vitamin K dependent glycoprotein in blood plasma. PC prevents the blood clotting in the blood vessels (anti-coagulant), dissolves the formed blood clots indirectly (anti-thrombosis), and prevents inflammation (anti-inflammation) (Esmon, et al., 1999).

When a person has a PC deficiency, blood clots may be formed inside blood vessels and these may cause thromboembolic symptoms, such as, lung embolism, heart attack, or stroke.

Anticoagulant drugs, such as warfarin (coumarin) and heparin, are used for the patient who has thromboembolic events. Warfarin is a vitamin K analog and blocks the production pathway of the vitamin K dependent blood coagulant factors (Hirsh, et al., 1998a). However, it frequently causes bleeding problem. Heparin is working as an anticoagulant catalyzing the inactivation of thrombin and activated human factor IX (Hirsh, et al., 1998b). It may also cause side effects of heparin induced thrombocytopenia (HIT) and bleeding problem.

In plasma, PC exists as an inactive form (zymogen) at a concentration of 4 µg/ml. It is activated only when it is needed and, therefore, PC does not have the side effects that warfarin and heparin have.

The frequency of homozygous and heterozygous PC deficiency together in all the thrombotic events is 10 %. The other 10 % of thrombosis in the thrombotic events occurs in people in surgery, pregnancy, oral contraceptive, etc. showing acquired PC deficiency (Tabernero, et al., 1991; Malm, et al., 1992; Allaart, et al., 1994).

Currently, from plasma, PC is purified using mouse monoclonal antibody as the ligand of affinity chromatography. Ceprotin<sup>TM</sup>, human plasma PC purified by the mouse monoclonal antibody (Mab) against PC (Schoppmann, et al., 2001), has been licensed in European countries for patients with a severe congenital PC deficiency (Baxter International, Inc., 2001). The cost of Ceprotin<sup>TM</sup> is \$78.5 per 100 µg.

To reduce the PC purification cost, single chain variable regions (mini-Mab) of the Mab against PC was developed by way of phage display method using human V-gene library (Wu, et al., 1998b). Thirteen recombinant *E. coli* HB2151 colonies, which produce the mini-Mab against PC, were provided by Dr. Michael Sierks at the Arizona State University. A preliminary study of the mini-Mab purification, the PC purification using the purified mini-Mab, and an economic analysis of PC production using mini-Mab compared with that using Mab were performed by Korah, et al. (2003).

In this dissertation, the results of three different studies are presented: (1) optimization of the mini-Mab production condition, (2) optimization of the mini-Mab purification process, and (3) the PC purification from Cohn Fraction IV-1 using the purified mini-Mab as ligand of the affinity chromatography.

## **CHAPTER II**

### **BACKGROUND**

#### **A. PROTEIN C**

Protein C (PC) is a vitamin K dependent glycoprotein and it is produced in the liver as the form of zymogen (Stenflo, 1976; Esmon, 1990; Dreyfus, et al., 1991). In human blood plasma, the typical PC concentration level is 4 µg/ml and the half-life of PC in blood plasma is 6-10 hours (Riess, et al., 1985; Miletich, et al., 1987; Okajima et al., 1990; Dahlback, 1995). PC is converted to an activated PC (aPC) by thrombin-thrombomodulin complex at the endothelial cells (Comp, et al., 1980; Marlar, et al., 1981; Esmon, 1990). aPC stimulates anti-coagulation by deactivating activated coagulants, factors V and VIII (Kisiel, et al., 1977). aPC also inactivate plasminogen activator inhibitor (Marlar, et al., 1982) allowing tissue plasminogen activator (tPA; antithrombotic agent) free. The free tPA converts plasminogen to plasmin and the plasmin degrades the formed blood clot (de Fouw, et al., 1988). aPC prevents inflammation by modulating inflammatory cytokine production and by blocking selectin-mediated neutrophil binding (Esmon, 1990). The half-life of aPC in plasma is 15 to 30 minutes (Esmon, 1990).

There are three forms of PC deficiencies, homozygous, heterozygous, and acquired PC deficiencies. Homozygous PC deficient patients are born with two abnormal copies of the PC gene (Miletich, et al., 1993). Homozygous PC-deficient patients,

usually newborn infants, have very little of the functional PC in blood and, therefore, have severe thrombosis throughout the body including microvascular thrombosis in brain and wide spread intravascular coagulation. Without immediate clinical treatment, these conditions ultimately cause death. The frequency of homozygous PC deficiency is about 1 in 250,000 to 500,000 births (Marlar, et al., 1990).

Heterozygous PC deficiency patients are born with a lower concentration of PC than that of normal people (Type 1) or with an abnormal copy of the PC gene (Type 2). Heterozygous PC-deficient patients are at risks of venous thrombotic events, including deep vein thrombosis, pulmonary embolism, stroke, and heart attack. Although they are not as urgent cases as those with the homozygous PC deficiency, the heterozygous PC deficiency exhibits symptoms in most individuals before 30 years of age (Sills, et al., 1984; Allaart, et al., 1995). The frequency of heterozygous PC deficiency is 0.1 to 0.5 percent of the general population (Miletich, et al., 1993).

Each year, approximately 1 in 1,000 people, who do not have any genetic PC deficiency, at the age of 20 and 50, are affected by venous thrombosis (Goldhaber, et al., 1994). 3-10 % of these groups have a lower PC concentration level than the normal (Makris, et al., 1997; Heijboer, et al., 1990). These acquired PC deficiencies are associated with surgery, pregnancy, the use of oral contraceptives, Coumarin therapy, liver diseases, or body immobilization (Makris, et al., 1997; Malm, et al., 1992; Tabernero, et al., 1991; Allaart, et al., 1994).

Successful treatments for homozygous PC deficient patients using fresh-frozen PC-containing plasma have been reported (Marlar, et al., 1990; Dreyfus, et al., 1991). Frequent infusions of the fresh-frozen plasma, however, result in hyperproteinemia (Sills,



et al., 1984). Ceprotin<sup>TM</sup>, human plasma PC concentrate without other clotting proteins has been licensed in European countries recently (Baxter International, Inc., 2001). Ceprotin<sup>TM</sup> is for purpura fulminans and coumarin-induced skin necrosis in patients with a severe congenital PC deficiency. Ceprotin<sup>TM</sup> costs approximately \$80 per 100 µg.

Activated PC can be used also for the treatment of sepsis, which is the systemic response to severe bacteria infection in the blood and is associated with multiple organ dysfunctions (Bone, et al., 1997). Sepsis has been reported as the leading causes of death in surgical intensive care units. Approximately 750,000 cases of sepsis occur in the United States each year (Bernard, et al., 2001). The mortality rate of severe sepsis is approximately 20 % within 6 months (Rangel-Frausto, et al., 1995). Recently, a recombinant human activated PC, Xigris<sup>TM</sup>, has been approved by the US Food and Drug Administration (FDA) to treat sepsis (Eli Lilly, 2001). The average wholesale prices of the 5 mg and 20 mg Xigris<sup>TM</sup> are \$210 and \$840, respectively. The price for the recommended 96-hour dose (~0.2 g) is \$7,700.

Therefore, it is very important to provide cheap PC to PC-deficient patients, sepsis patients, patients having various thrombo-embolic episodes.

## B. PC SOURCES

Currently available PC sources are human blood plasma (Schwarz, et al., 1990), mammalian cell culture medium (Grinnell, et al., 1990), and transgenic pig milk (Velandar, et al., 1992; Dalton, et al., 1997). Human blood plasma is a natural source and has less immunogenic reactions in the human body. The production level of recombinant human PC (rhPC) from mammalian cell culture medium was reported to be 20 to 30

$\mu\text{g/ml}/10^6$  cells/day from adenovirus transformed human kidney 293 cell lines (Grinnell, et al., 1990). The rhPC production level in transgenic pig milk was reported at 380  $\mu\text{g/ml}$  per hour from the mammary gland of a pig (Velandar, et al., 1992). However, the total recovery is only 24 % and PC production from transgenic pig milk requires many steps and large quantities of precipitation agents like polyethylene glycol (Wu, 2000; Wu, et al., 2002).

Commercially available cheap human plasma derived Cohn fraction IV-1 can be used as a volume reduced and enriched starting materials for producing pure PC (Velandar, et al., 1990).

Cohn, et al. have developed a method to fractionate many blood plasma proteins on a large scale, which were required in wartime (Creager, 1999). It was based on changing the solubility of the proteins using different pH values, temperatures, and ethanol concentrations. The plasma proteins were separated into five major fractions (Cohn, et al., 1946): as much as possible of fibrinogen in Fraction I,  $\gamma$ -globulins in Fraction II, lipid-bearing  $\beta$ -globulins in Fraction III,  $\alpha$ -globulins in Fraction IV, and albumins in Fraction V. To separate the proteins effectively, several methods were tried changing the ethanol concentration and pH (Figure 1). Cohn Fraction IV-1 is a byproduct of human serum albumin fractionation of blood plasma and usually discarded (Cohn, 1948).

The Cohn Fraction IV-1 is known to include over 90 % of PC in Plasma, and coagulating factors, such as factors II, VII, IX, and X (Velandar, et al., 1990; Wu, et al., 1998a).

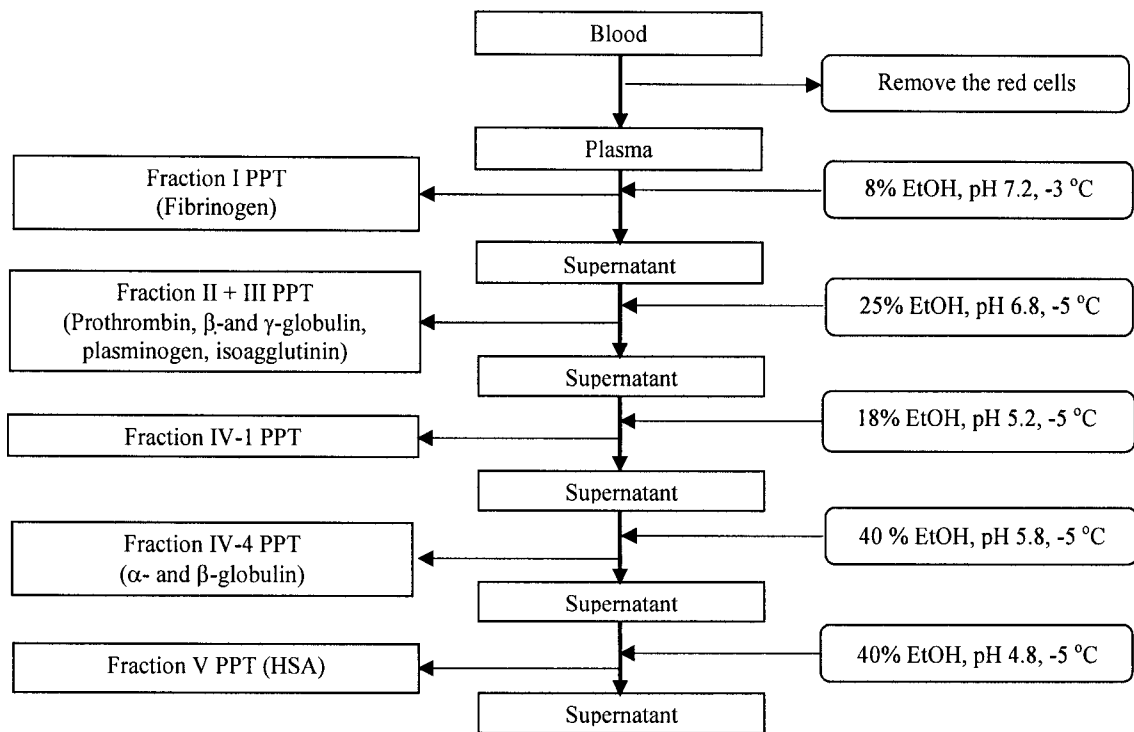


Figure 1. Schematic diagram of Cohn Fractionation.

## C. METHODS USED FOR THE PURIFICATION OF PC FROM PLASMA

### 1. Immuno-Affinity Chromatography

Although human blood plasma and its derivatives are good sources of PC, they contain several proteins homologous to PC. Many of these homologues are coagulants, such as, Factors II, VII, IX and X (Foster, et al., 1985; Furie, et al., 1988; Leytus, et al., 1986; Yoshitake, et al., 1985). Traditional chromatography methods other than immuno-affinity method, therefore, may not be employed for PC purification. PC has been purified using immuno-affinity chromatography (Nakamura, et al., 1987; Schoppmann, et al., 2001; Schwarz, et al., 1990).

Nakamura, et al. (1987) produced monoclonal antibodies and obtained 59.3 % of PC recovery by immunoaffinity chromatography. They reported also that 70 % of PC

was adsorbed on their monoclonal antibody using commercially available prothrombin complex concentrate, an eluate of anion exchange chromatography of human blood plasma in single step purification (Nakamura, et al., 1987).

Velander, et al. (1990) have reported PC purification from an eluate of DEAE anion exchange chromatography of reconstituted Cohn Fraction IV-1. 2 mg of IgG was immobilized on 1 ml matrix. Using approximately 40 ml of the reconstituted Cohn Fraction IV-1 and 4 ml of the matrix, 25 % overall recovery of PC was obtained using direct immunopurification (Velander, et al., 1990).

Kang, et al. (1992) developed a method for estimating the immunoaffinity PC purification performance using an affinity chromatography to estimate both the time and the cost of a large-scale PC purification process. The points to consider the mass production of PC are Mab adsorption efficiency on gel matrices, adsorption efficiency of PC to the immobilized Mab, and the specific activity of the eluted PC. In addition, it was reported that for the scale up of PC purification processes, pressure tolerance of the gel matrices and production rate should be considered.

## 2. IMAC

The cost of Mab production using animal cell culture is very high. Hence, PC purified using Mab is very expensive. Therefore, a much cheaper method than immunoaffinity chromatography, immobilized metal affinity chromatography (IMAC), was investigated to purify PC from Cohn Fraction IV-1 (Wu, et al., 1998a; 1999).

PC purification from prothrombin, a major coagulant factor in plasma, was studied using the mixture of PC and prothrombin. IDA- copper ion was used and 95.5 %

of active PC was effectively recovered (Wu, et al., 1999). Therefore, it was reported that copper IMAC may be cost effective method to purify PC from prothrombin instead of using immunoaffinity chromatography.

For the PC purification from Cohn Fraction IV-1 using iminodiacetic acid (IDA) and copper ion, 84 % of PC recovery was obtained (Wu, et al, 1998a). However, the eluate included many contaminants based on gel electrophoresis, indicating that PC in Cohn Fraction IV-1 may not be separated by one step IMAC process (Wu, 2000)

#### D. PC HOMOLOGUES

Vitamin K dependent homologous proteins are factors II, VII, IX, and X as well as PC, protein S, and protein Z and all are produced in liver (Broze, et al., 1984). Factors II, VII, IX, and X play a key role in blood clotting (Kisiel, et al., 1977; Marlar, et al., 1982; Esmon, 1990). Protein S is acting as a cofactor of the activated PC to enhance the inactivation of the activated factor V and VIII (Griffin, et al., 1999). Protein Z is considered to suppress thrombus formation inhibiting the activated factor X (McQuillan, et al., 2003).

The properties of vitamin K-dependent proteins were compared in Table 1. The molecular weights and the isoelectric points of the proteins are closely distributed at the range of between 50,000 and 72,000 kDa and between 4.0 and 5.5, respectively. Therefore, the separation of PC from these homologous proteins is a challenge.

After separation of PC, several methods including gel electrophoresis, enzyme linked immunosorbent assay (ELISA), and the activity test may be required to characterize the purified PC.

Table 1. Properties of vitamin K-dependent proteins.

Proteins	Molecular Weight	Function	Conc. in plasma (µg/ml)	Half life in plasma (hours)	Level Required for Hemostasis	Isoelectric point	Extinc. Coeff. ( $E_{1cm, 280nm}^{1\%}$ )	Reference
Protein C	62,000	Anticoagulant	3.9-5.9	6-10	> 60 %	4.4 – 4.8	14.5	[24], [28], [33], [56], [66]
Protein S	69,000	PC cofactor	20-35	15 (rabbit)	> 50 %	5.0 - 5.5	9.5	[24], [28], [33], [56]
Protein Z	62,000	-	2.9	60	-	-	12.0	[19], [24], [60]
Factor II	72,000	Coagulant	80-90	48-120	20-40 %	4.7 – 4.9	13.8	[24], [33], [34], [54], [56]
Factor VII	50,000	Coagulant	0.47	4-7	10-20 %	4.8 – 5.1 (bovine)	13.9	[8], [17], [24], [33], [56], [100]
Factor IX	57,000	Coagulant	4-5	20-24	25-30 %	4.0 – 4.6	13.2	[24], [33], [34], [56], [91]
Factor X	59,000	Coagulant	6.4	24-40	10-20 %	4.8 – 5.0	11.6	[24], [33], [34], [55], [56]

#### E. MINI-ANTIBODY SPECIFIC AGAINST PC

Immunoglobulin (IgG) is produced from B cell in the spleen and consists of four polypeptide chains (Figure 2). In an IgG molecule, two heavy chains and two light chains are held together by disulfide bonds. The antigen-binding site is at the amino end of each variable fragment (Lu, et al., 1996). The antigen recognition region consists of one variable region in one heavy chain ( $V_H$ ) and one variable region in one light chain ( $V_L$ ).

The heavy chain and light chain V-genes are shuffled at random and cloned for displaying as single chain variable fragments (scFvs) on the surface of filamentous phage to make the phage antibody library containing V-genes, and the phage-antibody fragments of the library are selected by binding to antigen. In addition, scFv with high specificities of binding to human self-antigens can be made from the same phage library

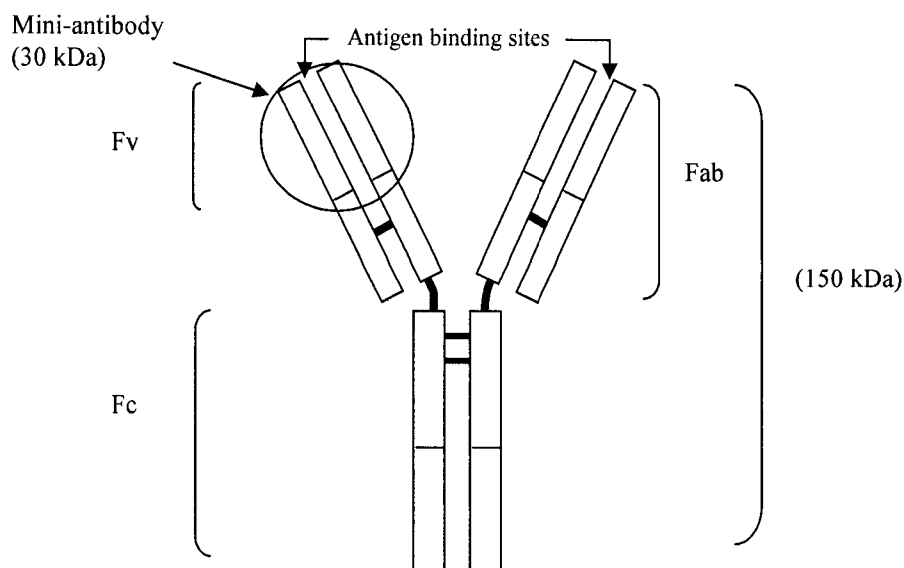


Figure 2. Structure of Mab.

(Griffiths et al., 1993; Marks, et al., 1991). The mini-Mab against PC in this study was developed using the same V-genes (Wu, et al., 1998).

The phage library including V-genes specific to PC is isolated by a two-step selection procedure from the library (Wu, et al., 1998b). First, undesired coagulant factors, Factor IX or Factor X, which are homologous proteins to PC, are coated on a solid support - a micro titer plate used for enzyme linked immunosorbent assay (ELISA).

Advantages of producing mini-Mabs using the recombinant *E. coli* compared with Mabs include less contamination problems during production, less shear sensitivity of the host cell, less expense, and easier scale-up.

## F. PREVIOUS STUDY ON PC PURIFICATION USING MINI-MAB

Mini-Mab purification and PC purification using PC mini-Mab were initiated by Wu, et al. (1998b). Korah, et al. (2003) carried out preliminary, quantitative studies on mini-Mab purification and PC purification using the purified mini-Mab. The following studies were performed by Korah.

Mini-Mab Production and Purification. The concentration of IPTG as an inducer in the mini-Mab production media was optimized from 1 mM to 0.1 mM. Mini-Mab was purified from the protein A immobilized CNBr-Sepharose chromatography. The 18 % of mini-Mab purification efficiency was obtained using an elution pH 3.0 of the glycine buffer. The metal ions of IMAC have affinity to histidine, cysteine, and tryptophan. Mini-Mab may contain these metal ion binding residues on the amino acid side chains of its surface. Therefore, mini-Mab purification was tested using IMAC column. The commercially available HiTrap Chelating IMAC column with a pre-immobilized chelator was used. Metal ions such as  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$ , and  $\text{Fe}^{2+}$  were applied to the column.  $\text{Cu}^{2+}$  and  $\text{Co}^{2+}$  were leached out during the mini-Mab adsorption at pH 7.4. Fe-IMAC showed only 0.3 % of mini-Mab purification yield at pH 7.4. When Ni-NTA was used at pH 6.4, using the purified mini-Mab, 40% of mini-Mab purification yield was obtained.

PC Purification. PC purification using the pure sample was performed on mini-Mab immobilized gel matrices. CNBr activated sepharose and monoaldehyde-agarose (Actigel<sup>TM</sup> ALD) matrices were tested. Immobilization efficiency showed approximately 80 % and 45 % for the CNBr activated sepharose matrix and Actigel, respectively. PC purification efficiency using the CNBr activated sepharose and the Actigel showed approximately 6 % and 10 %, respectively.



Economic Analysis of PC Production. PC production cost using between mini-Mab and Mab, Korah (2003) were compared in terms of the costs of antibody production, antibody purification, and PC purification. The specific production cost of mini-Mab was evaluated 500 times lower than that of Mab's. Based on mini-Mab purification using IMAC column, the yield of Mab purification (70 %) was evaluated 1.75 times higher than mini-Mab's (40 %; Korah, 2002). PC purification yield using Mab (70 %) was evaluated 7 times higher than mini-Mab's (10%). Based on the data obtained from the antibody production, antibody purification, and PC purification, Korah estimated that PC purification using the mini-Mab is 40 times cheaper than using regular Mab.

## CHAPTER III

### MATERIALS, INSTRUMENTS, AND METHODS

#### A. MATERIALS

##### 1. Mini-Mab Production

Thirteen recombinant *E. coli* HB2151 colonies that contain the PC mini-Mab producing gene were provided by Dr. Michael Sierks at the Arizona State University. The *E. coli* culture media was made using Trypton, NaCl, glucose, phenylmethylsulfonyl fluoride (PMSF), ampicillin (Sigma-Aldrich, Inc.; St. Louis, MO), and yeast extract (Bacto Laboratories; Liverpool, Australia). Isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG; ICN Biomedical, Inc.; Irvine, CA) was added into the production media to induce the mini-Mab expression. Lactose was added (Sigma-Aldrich, Inc.) and sucrose, Triton X-100, and phospholipase A2 (Sigma-Aldrich, Inc) for the study of mini-Mab release from *E. coli* cells. *E. coli* cells were separated by a centrifuge in centrifuge tubes (Greiner Bio-one; Longwood, FL). The supernatant was concentrated using Centricon, a 19 ml ultrafiltration filter (Millipore; Schwalbach, Germany).

##### 2. Mini-Mab Purification

Protein A immobilized CNBr-activated Sepharose<sup>TM</sup> CL-4B (Protein A Sepharose<sup>TM</sup>), glycine, and citric acid were purchased from Sigma-Aldrich. Glass Econo-Column<sup>®</sup> chromatography column (Bio-Rad; Hercules, CA) was used to pack the

gel matrix. Slide-A-Lyzer<sup>®</sup> Dialysis Cassette (Pierce; Rockford, IL) with 10,000 Da molecular weight cut off was used for the buffer dialysis.

### 3. PC Purification

PC and Cohn Fraction IV-1 paste were provided by the American Red Cross (Rockville, MD) and human blood factors II, VII, IX, and X were purchased from Innovative Research, Inc. (Southfield, MI). Normal hydroxysuccinimide (NHS)-activated Sepharose<sup>™</sup> 4 Fast Flow (NHS Sepharose<sup>™</sup>) and diethylaminoethyl (DEAE) Sepharose<sup>™</sup> Fast Flow (DEAE Sepharose<sup>™</sup>) were purchased from Amersham Biosciences (Piscataway, NJ). Actigel ALD<sup>™</sup> (Actigel<sup>™</sup>) and Epoxy Activated Ultraflow 4<sup>™</sup> (Epoxy Activated<sup>™</sup>) were from Sterogene (Carlsbad, CA). A Supor<sup>®</sup>, membrane filter papers with 47 mm diameter and 0.2, 0.45, and 0.8  $\mu$ m pore sizes were obtained from Gelman Sciences (Ann Arbor, MI) to filter buffer and Cohn paste solution.

### 4. Gel Electrophoresis

Tris base, lauryl sulfate, glycerol, bromophenol blue, methanol, Brilliant Blue R, acrylamide solution, ammonium persulfate, tetramethylethylenediamine (TEMED) and acetic acid (Sigma-Aldrich) were used for the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SigmaMarker<sup>™</sup> standard molecular marker (Sigma-Aldrich; myosin, 205 kDa;  $\beta$ -galactosidase, 116 kDa; phosphorylase b, 97 kDa; fructose-6-phosphate kinase, 84 kDa; bovine serum albumin, 66 kDa; glutamic dehydrogenase, 55 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20 kDa;  $\alpha$ -lactalbumin, 14.2

kDa; and aprotinin, 6.5 kDa) was used for the comparison of the molecular weights of proteins on the SDS-PAGE.

## 5. Enzyme Linked Immunosorbent Assay (ELISA)

96 well microtiter plates (Nalgen Nunc International; Roskilde, Denmark) were used for ELISA for the measurement of the concentrations of the mini-Mab, PC, vitamin K dependent coagulation factors, and human serum albumin (HSA). 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris base),  $\text{NaHCO}_3$ ,  $\text{Na}_2\text{CO}_3$ , phosphate buffered saline (PBS), Tween 20, horseradish peroxidase-conjugated goat anti mouse IgG, o-phenylenediamine dichloride (OPD) solution containing urea hydrogen peroxide, human serum albumin, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. For the mini-Mab ELISA, anti-c-myc antibody (9E10) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). For the ELISA of PC and the other factors, Rabbit polyclonal factor II antibody was purchased from Biomeda Corporation (Foster city, CA) and mouse monoclonal factor II antibody, from Enzyme Research Laboratories (South Bend, IN). Rabbit polyclonal factor VII antibody was obtained from Novus Biologicals, Inc. (Littleton, CO), and goat polyclonal factor X antibody and goat polyclonal human serum albumin antibody, from US biological (Swampscott, MA). Mouse monoclonal human serum albumin antibody was purchased from Biodesign International (Saco, ME). Rabbit polyclonal antibodies for PC and factor IX, mouse monoclonal antibodies against PC and factors VII, IX, and X were obtained from Sigma-Aldrich.

## 6. Protein Assay

The total protein was quantified using Bicinchoninic Acid (BCA\*™) protein assay reagents from Pierce (Rockford, IL).

All other chemicals, unless otherwise specified, were purchased from Sigma-Aldrich.

## B. INSTRUMENTS

### 1. Mini-Mab Production

Antibodies, *E. coli* cell lines, and purified samples were stored in a -70 °C freezer (Resco; Ontario, Canada). An autoclave (Sterilmatic; Market Froge; Everett, MA) was used to sterilize the media by heat or a disposable sterile syringe filter (Corning Glass Works; Corning, NY) was used to sterile the media. A laminar hood (Purifier Class II Biosafety Cabinet; Labconco Corporation; Kansas City, MO) was used for the control of contamination while the *E. coli* cells are inoculated in the media. *E. coli* was cultured in a shaking incubator (C24 incubator shaker; New Brunswick Scientific; Edison, NJ). Accumet pH Meter (Fischer Scientific; Pittsburgh, PA) was used to measure the pH. *E. coli* cell density and the protein concentration were measured using a DU SERIES 500 Spectrophotometer (Beckman Instruments, Inc.; Fullerton, CA). The produced mini-Mab and the purified PC were quantified using an ELISA plate reader (Bio-Rad; Hercules, CA). The incubation temperature for protein quantification and ELISA was controlled in Isotemp Incubator (Fischer Scientific).

## 2. Mini-Mab and PC Purification

A Marathon 3200<sup>®</sup> general-purpose centrifuge (Fischer Scientific) was used for *E. coli* cell separation and the ultra-filtration. The purification of the mini-Mab and PC is performed using FRAC-100 (Amersham Biosciences; Piscataway, NJ) and its accessories, peristaltic pump P-1, Control Unit UV-1, Optical Unit UV-1, and the recorder REC 101. Chromatography columns were purchased from Bio-Rad (Hercules, CA). SDS-PAGE was carried out using a Mini-Protean 3 Cell/ PowerPac Basic System (Bio-Rad) and its accessories, combs, plates, and casting accessories for 10-well.

## C. METHODS

### 1. Mini-Mab Production

Culture media for the *E. coli* cell growth and mini-Mab production were prepared. 2XTY media (Trypton: 16 g/l; yeast extract: 10 g/l; NaCl: 5 g/l) was autoclaved at 121 °C for 20 minutes. Glucose stock solution at a concentration of 200 g/l was filtered using syringe filters of 0.45 µm pore size (Corning Glass Works; Corning, NY), and applied to a flask to adjust the final concentration of 1% for the cell growth media and 0.1 % for the mini-Mab production media. Ampicillin stock solution at a concentration of 100 mg/ml was applied to the media to make the final concentration of 100 µg/ml.

The compositions of the cell growth and the mini-Mab production media are as follows:

- (1) Cell growth media: 2XTY media, 1 % glucose, and 100 µg/ml of ampicillin.
- (2) Mini-Mab production media: 2XTY media, 0.1% glucose, 100 µg/ml of ampicillin, and 1 mM of IPTG.

*E. coli* colonies from the cell stock were added to the cell growth media and cultured at 37 °C in a shaking incubator at 300 rpm. When the optical density (O.D.) of the culture media reached 0.9 at the wavelength of 600 nm, the cells were separated using a centrifuge at 4000 rpm for 20 minutes. The separated cells from the cell growth media were transferred to the mini-Mab production media and cultured in the shaking incubator at 300 rpm for 24 hrs at 30 °C. After the mini-Mab production, cells were separated using the centrifuge at 4000 rpm for 20 minutes. The concentration of mini-Mab in the supernatant is measured using ELISA. To make the *E. coli* cell stock, *E. coli* cells were grown in the cell growth media until the optical density (O.D.) of the media becomes 0.9, and the *E. coli* cells were separated using the centrifuge at 4000 rpm for 20 minutes. The separated cells were transferred to 20% glycerol solution in the laminar hood and stored in a deep freezer (-70 °C). Whenever needed, the *E. coli* cell stock was thawed and inoculated to the media in the laminar hood.

## 2. Mini-Mab Purification

The mini-Mab in the production media supernatant was purified using Protein A Sepharose<sup>TM</sup> following the manufacturer's instruction. Protein A Sepharose<sup>TM</sup> matrix was suspended with 0.02 M NaH<sub>2</sub>PO<sub>4</sub>-0.15 M NaCl solution (pH 8.0) overnight at 4 °C to swell. The matrix was degassed using vacuum pump for an hour at 200 mm Hg. The matrix suspension was transferred into a chromatography column and was allowed to settle for an hour. Then, it was washed with 20-bed volumes of 0.02 M NaH<sub>2</sub>PO<sub>4</sub>-0.15 M NaCl solution.

After the protein A column is prepared, the supernatant from the production media was applied into the column at the linear velocity of 0.015 cm/s. After the adsorption process is completed, any unbound protein was washed using 0.02 M  $\text{NaH}_2\text{PO}_4$ -0.15 M NaCl solutions until the UV reading reached the base line. The bound mini-Mab was eluted with 0.1 M glycine buffer (pH 3.0) or 0.1 M citric acid buffer (pH 3.0). Fractions were collected, and neutralized with 0.1 M NaOH. The column was washed with 20-bed volume of 0.02 M  $\text{NaH}_2\text{PO}_4$ -0.15 M NaCl solution following 0.5 M NaCl solution. The column was re-equilibrated with 20-bed volume of 0.02 M  $\text{NaH}_2\text{PO}_4$ -0.15 M NaCl solutions for the next use. The amounts of the mini-Mab in the supernatant, eluted fractions, and other solutions were measured using ELISA.

### 3. PC Purification

Actigel ALD<sup>TM</sup> matrix. Mini-Mab coupled Actigel<sup>TM</sup> matrix was prepared following the manufacturer's instruction. Approximately 1 ml of the matrix was applied to a sintered glass filter and washed with 3-bed volumes of 0.1 M PBS buffer (coupling buffer; pH 6.9). The gels were suspended with 1 ml of the coupling buffer. Predetermined amount of the mini-Mab in coupling buffer was added to the gel suspension. 1 M  $\text{NaCNBH}_3$  (coupling solution) was added to the gel suspension and mini-Mab mixture at the final concentration of 0.1 M. The total volume of the mixture was adjusted to 3.5 ml with 0.1 M PBS buffer. The coupling reaction was performed by a shaking incubator at 100 rpm and 30 °C for 5 hours. After coupling, the supernatant was removed using a sintered glass filter with 150 micron of pore size.



The matrix was washed with 3 bed-volume of adsorption/washing buffer (0.02 M sodium citrate-0.08 M NaCl at pH 6.0) following washing with 10-bed volume of 0.5 M of NaCl to remove non-covalently bound mini-Mab from the matrix. The filtrate was used to calculate the coupling yield. It was then degassed under vacuum for an hour at 200 mm Hg. The gels were packed in a column. The matrix was equilibrated with 10-bed volume of 0.02 M sodium citrate-0.08 M NaCl (pH 6.0).

0.02 M sodium citrate-0.08 M NaCl at pH 6.0 (adsorption/washing buffer) and 0.1 M sodium carbonate buffer-0.15 M NaCl at pH 10.0 (elution buffer) were chosen for PC purification following the PC purification protocol of Kang, et al. (1992). When the column for the PC purification was ready, 20 µg of pure PC was applied to the column. 10 minutes was allowed for satisfactory adsorption reaction. The column was then washed with 10-bed volume of adsorption/washing buffer at the linear velocity, 0.015 cm/s. After the washing was complete, the elution buffer was applied at the velocity of 0.015 cm/s until the UV reading returned to the base line. The pH of the eluted PC was immediately neutralized by adding 3.0 M HCl. The column was then washed with the 10-bed volume of adsorption/washing buffer and 0.5 M NaCl following the 10-bed volume of adsorption/washing buffer.

NHS-activated Sepharose<sup>TM</sup> 4 Fast Flow. Coupling of the mini-Mab was performed by the manufacturer's instruction. 1 ml of NHS-activated Sepharose<sup>TM</sup> 4 Fast Flow (NHS Sepharose<sup>TM</sup>) matrix was washed on a sintered glass filter with 10 ml of 1 mM HCl at 4 °C. The matrix was suspended in 10 ml of 0.2 M NaHCO<sub>3</sub>-0.5M NaCl (pH 8.2; coupling buffer). Immediately afterwards, predetermined amount of the purified mini-Mab was added to the matrix suspension. The total mixture volume was adjusted to

6 ml. Incubation was performed by a shaking incubator at 100 rpm and 30 °C, for 5 hours. After coupling, the supernatant was removed and 6 ml of 0.1 M Tris-HCl (pH 8.0; blocking buffer) was added to the matrix, was reacted for additional 4 hours at room temperature. The matrix was packed in a chromatography column. To remove the non-covalently bound mini-Mab, the gel in the column was washed with 25-bed volumes of each PBS buffer (pH 7.3), PBS-1 M NaCl, cold water, 0.1 M Na<sub>2</sub>CO<sub>3</sub>-0.5 M NaCl (pH 11), 0.1 M sodium acetate (0.5 M NaCl; pH 4), 0.1 M Na<sub>2</sub>CO<sub>3</sub>-0.5 M NaCl (pH 11), 0.1 M sodium acetate (0.5 M NaCl; pH 4) and again PBS respectively. The mini-Mab amount in the filtrate was quantified by ELISA to obtain the coupling efficiency.

Epoxy Activated Ultraflow™. Mini-Mab coupled Epoxy Activated Ultraflow™ matrix was prepared following the manufacturer's instruction. Approximately 1 ml of the gel matrix was applied to a sintered glass filter and washed with 5 ml of deionized (DI) water. Predetermined amount of the mini-Mab was dissolved in 2 ml of 0.1 M sodium phosphate buffer (pH 10; coupling buffer). The pre-washed matrix was added to the mini-Mab solution. The solution of the matrix and mini-Mab was mixed in a shaking incubator at 100 rpm and 25 °C, for 18 hours. After coupling, the supernatant was removed and 1.5 ml of 50 mM of ethanolamine (pH 8.5; blocking buffer) was added to the complex of the mini-Mab and the matrix and reacted for additional 4 hours at room temperature. To remove the non-covalently bound mini-Mab, the gel in the column was washed with 25-bed volumes of DI water and 3-bed volumes of adsorption/washing buffer (0.02 M sodium citrate-0.08 M NaCl at pH 6.0) following washing with 10-bed volume of 0.5 M of NaCl. The gel matrix was equilibrated with 10-bed volume of the adsorption/washing buffer.

#### 4. ELISA

ELISA for the mini-Mab quantification. The ELISA protocol was originally developed by Wu, et al. (1998) and optimized by Korah, et al. (2003).

The washing buffer used for this assay is phosphate buffered saline with 0.05% Tween 20 (PBST, 0.01 M PBS, 0.138 M NaCl, 0.0027 M KCl, pH 7.4), dilution/adsorption buffer was PBS, and the blocking buffer was PBST with 1 % BSA.

- (1) Coat each well of a 96 well plate with 100 µl of PC at a concentration of 1 µg/ml in adsorption buffer except the first column (control).
- (2) Incubate the plate overnight at 4 °C.
- (3) Wash each well with the PBS buffer (250 µl/well) three times.
- (4) Apply the blocking buffer (250 µl/well) in each well and incubate the plate for 90 minutes at room temperature.
- (5) Wash each well with the washing buffer three times.
- (6) Apply 100 µl of the dilution buffer to all wells except the wells on the first (control) and the second columns. Apply 200 µl of the mini-Mab standard, as well as the samples, to the respective rows in the second column and perform a ½ serial dilution.
- (7) Incubate the plate at room temperature for two hours.
- (8) Wash each well with the washing buffer three times.
- (9) Apply 100 µl/well of 9E10 antibody at a concentration of 1 µg/ml in PBS to all wells except first column.
- (10) Incubate the plate for two hours at room temperature.
- (11) Wash each well with the washing buffer three times.

- (12) Prepare horseradish peroxidase (HRP) conjugated goat anti-mouse IgG at a 1:1000 dilution in PBS and add 100 µl/well.
- (13) Incubate the plate for 30 minutes at room temperature.
- (14) Wash each well with each washing buffer and PBS three times respectively.
- (15) Add 100 µl/well of OPD solution.
- (16) Incubate the plate in a dark room for 30 minutes at room temperature.
- (17) Read the absorption of the wells at 450 nm using the ELISA plate reader. Calculate the concentration of the mini-Mab in the sample using the standard.

ELISA for quantification of PC and other factors. ELISA was based on the protocol developed by many researchers (Boyer, et al., 1984; Soria, et al., 1985; Suzuki, et al., 1985) and this ELISA protocol was optimized by the American Red Cross (Wu, 2000b).

The washing buffer used for this assay is Tris buffered saline (TBS, 12.5 mM Tris buffer, 0.05 M NaCl, pH 7.2) with 0.05% Tween 20 (TBST), dilution buffer was TBS, and the blocking buffer was TBS with 0.1% BSA for the PC and other factors or 4 % fish serum for human serum albumin. Buffer for adsorption on the wells was 0.1 M NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> at pH 9.6.

- (1) Coat the wells with 100 µl of the polyclonal antibody against the factor in the adsorption buffer at the concentration of 2 µg/ml.
- (2) Incubate the plate overnight at 4 °C.
- (3) Wash each well with the washing buffer (250 µl/well) three times.

- (4) Block each well with the blocking buffer (250  $\mu$ l/well) for 90 minutes at 37 °C in an incubator.
- (5) Wash each well with the washing buffer three times.
- (6) Apply 100  $\mu$ l of the dilution buffer to all wells except the wells on the first (control) and the second columns. Apply 200  $\mu$ l of PC or other factors as standard, as well as the samples, to the respective rows in the second column and perform  $\frac{1}{2}$  serial dilutions.
- (7) Incubate the samples at 37 °C in an incubator for 90 minutes.
- (8) Wash each well with the washing buffer three times.
- (9) Apply 100  $\mu$ l/well of the mouse monoclonal antibody for the respective factor at a concentration of 1  $\mu$ g/ml.
- (10) Incubate the plate for 90 minutes in an incubator at 37 °C.
- (11) Wash each well with the washing buffer three times.
- (12) Prepare goat anti-mouse IgG peroxidase conjugate at a 1:1000 dilution in the dilution buffer and add 100  $\mu$ l/well.
- (13) Incubate the plate at room temperature for 30 minutes.
- (14) Wash each well with the washing buffer at least six times.
- (15) Add 100  $\mu$ l/well of the OPD solution.
- (16) Incubate the plate in a dark room at room temperature for 30 minutes.
- (17) Read the absorption of wells at 450 nm using an ELISA plate reader. Obtain the concentration of PC or other factors in the sample using the standard.

## 5. Protein Assay

Bicinchoninic Acid (BCA\*<sup>TM</sup>) protein assay was used to quantify the total protein in the sample. A 100  $\mu$ l of protein sample was added to 2 ml of the BCA\*<sup>TM</sup> Protein Assay Reagent. The solution was mixed and was incubated at 37 °C for 30 minutes. After the incubation, the solution was cooled to room temperature for an hour, and the absorbance was measured at 562 nm. From the optical density of the sample, the concentration of protein was calculated using BSA as a standard.

Purified mini-Mab can be optically quantified using a spectrophotometer reading O.D. at 280 nm. The extinction coefficient of variable light chain of human Immunoglobulin G (IgG) was known to be  $E_{280\text{nm}}^{0.1\%} = 1.16$  (Fasman, 1989) but the extinction coefficient of the mini-Mab is not known.

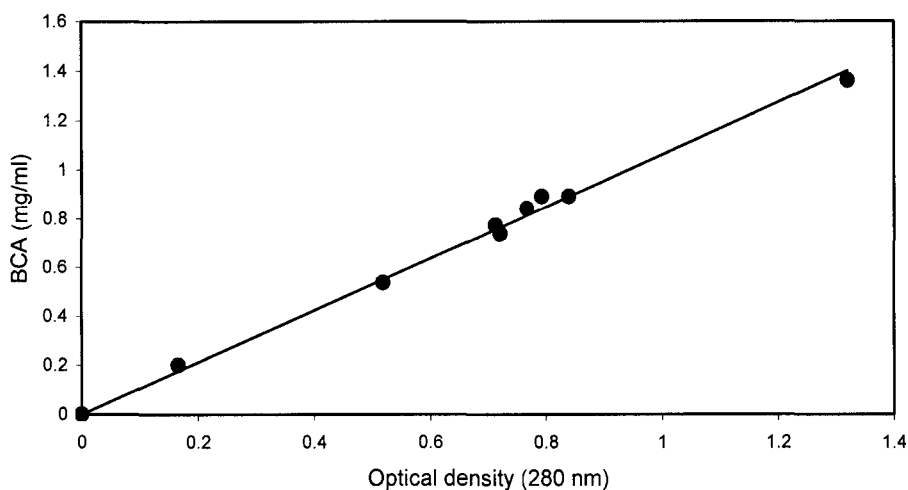


Figure 3. Standard curve for the purified mini-Mab concentration. BSA was used as a reference material for BCA protein assay. The incubation temperature and time were 37 °C and 30 minutes respectively.

Therefore, the amount of the mini-Mab by the BCA\*™ was correlated with the spectrophotometer reading at 280 for the purified mini-Mab (Figure 3). The extinction coefficient of the mini-Mab was estimated as  $E_{280\text{nm}}^{0.1\%}=0.94$ .

## 6. SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the manufacturer's instruction. The separation gel was made with 30% acrylamide solution, 20% ammonium per sulfate, water, TEMED, and Tris HCl buffer at pH 8.8. The protein was denatured in SDS solution at 100 °C for 5 minutes. Once the gel is ready, the 20 µl of each sample and the molecular marker were applied to wells respectively. The amount of protein sample in each well was at least 1 µg. The molecular weight was obtained by comparing the positions of the molecular markers that shows bands of known molecular weight.

## 7. Pretreatment of Cohn Fraction IV-1 for the PC Purification

The preparation of Cohn Fraction IV-1 sample was carried out based on the protocol by Wu (2000) with the modifications of the centrifugation speed using 4000 rpm instead of using 8000 rpm and dilution ratio of Cohn paste to buffer at 1 to 20 instead of at 1 to 30. The Cohn paste was dissolved in sodium citrate buffer (pH 6.0) at a ratio of 1 to 20 (weight to volume ratio) at 4 °C for 5 hours with constant stirring using a magnetic stirrer. Then the solution was centrifuged at 4000 rpm for 20 minutes and the supernatant was filtered using three filters with different pore sizes, such as, 0.8, 0.45, and 0.2 µm, respectively, which were found to be effective to filter the Cohn Fraction IV-1 solution

by Lee in our lab. The filtrate was used as the source material for the PC purification using the mini-Mab.

#### 8. Ion Exchange Chromatography of Cohn Fraction IV-1 Using DEAE Sepharose<sup>TM</sup> Fast Flow

Following the manufacturer's instruction, DEAE Sepharose<sup>TM</sup> Fast Flow (DEAE Sepharose<sup>TM</sup>) matrix was prepared. Approximately 1 ml of the gel matrix was applied to a sintered glass filter and washed with 5 ml of deionized (DI) water and degassed for one hour, and packed in a chromatography column. Then, the matrix was equilibrated with 10-bed volumes of 25 mM sodium citrate buffer (pH 6.0). Approximately 20 g of Cohn Fraction IV-1 was dissolved into 400 ml of 0.02 M sodium citrate buffer at pH 6.8 at 4 °C with magnetic stirring for approximately three hours. The Cohn Fraction IV-1 solution was then centrifuged at 4000 rpm for 20 minutes. The supernatant was adjusted to pH 6.0 and then applied to the DEAE Sepharose<sup>TM</sup> at a flow rate of 0.004 cm/s. The column was then washed with 0.025 M sodium citrate buffer (pH 6.0) using 50-bed volumes. The column was then washed with 20 column volumes of 0.02 M 4-morpholineethanesulfonic acid (Mes) including 0.17 M NaCl (pH 6.0) using about 150-bed volumes. Then, the fractions were eluted by 3- to 4-bed volumes of elution buffer including 0.02 M Mes and 0.37 M NaCl at pH 6.0. For the matrix regeneration, 0.02 M Mes including 2 M NaCl at pH 6.0 was used.



## CHAPTER IV

### RESULTS AND DISCUSSION

Three main studies performed were: optimization of PC mini-Mab production condition; mini-Mab purification using protein A Sepharose; and PC purification from Cohn Fraction IV-1 using the purified mini-Mab. Experimental results presented in this dissertation are from at least two sets of experiments, unless specified otherwise.

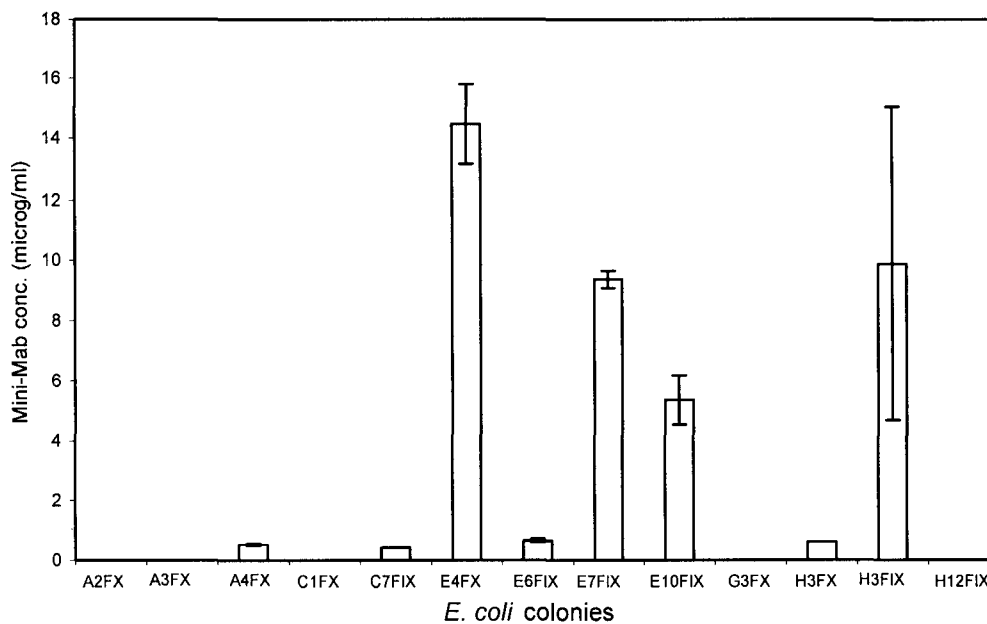
#### A. PROCESS OPTIMIZATION OF THE MINI-MAB PRODUCTION

##### 1. Selection of the Highest Mini-Mab Producing *E. coli* Colony

To produce the mini-Mab using *E. coli*, there are two stages of bioprocesses: a stage of *E. coli* growth and then a stage of the mini-Mab production by the *E. coli*.

The original protocol for the mini-Mab production, which was provided by Sierks, was with 2XTY media including 1 % of glucose; at 37 °C; and at a rate of shaking 300 rpm for the growth of the colony and with 2XTY media including 1 mM of IPTG, 1 mM PMSF, and 0.1 % of glucose; at 30 °C; at a rate of shaking 300 rpm; and for 24 hours of the production time for the mini-Mab production (Wu, et al., 1998b).

Thirteen *E. coli* colonies producing mini-Mab against PC, A2FX, A3FX, A4FX, C1FX, C7FIX, E4FX, E6FIX, E7FIX, E10FIX, G3FX, H3FX, H3FIX, and H12FIX were tested to select the colony producing the largest quantity of the mini-Mab.



**Figure 4.** Mini-Mab production levels by thirteen *E. coli* colonies.

[Experimental conditions: 30 °C, initial pH 7.0, and 0.1 % glucose]

After the completion of the production, the amount of the mini-Mab in the supernatant was quantified by ELISA (Figure 4). Among 13 colonies, E4FX, E7FIX, and H3FIX colonies produced much more than others, approximately 10 ~ 15 µg/ml of the mini-Mab in the supernatant. The production by E4FX was the greatest and was selected for further studies.

## 2. Glucose Concentration

Since the mini-Mab is produced using lac operon in *E. coli* cells, high concentration of glucose in the production medium inhibits the mini-Mab production by catabolic inhibition (Rousch, et al., 1998). Therefore, the effect of the glucose

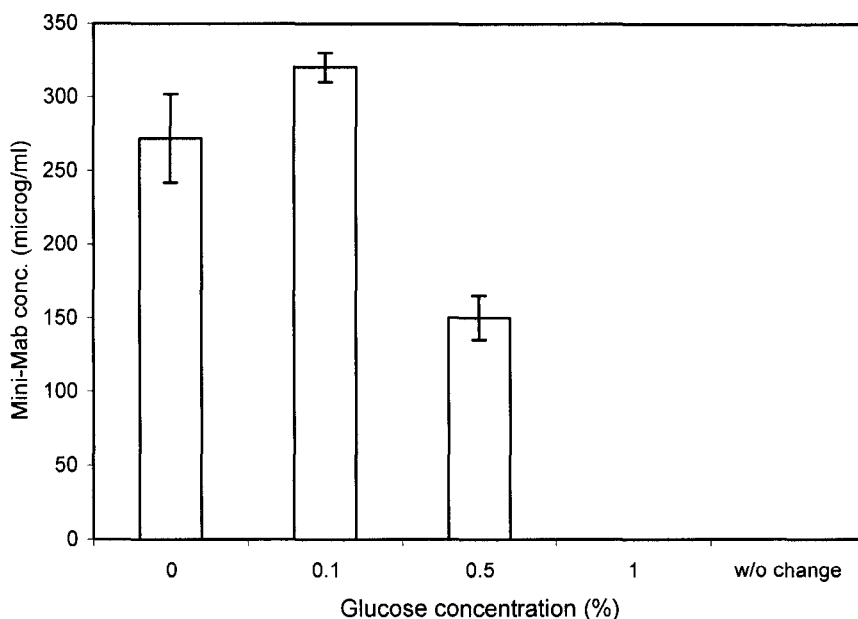


Figure 5. The effect of glucose concentration on mini-Mab production.

[Experimental conditions: 30 °C and initial pH 7.0]

concentration in the production media on the mini-Mab production was examined at various glucose concentrations, while other media composition remained constant (Figure 5).

When the glucose was not added to the production media, mini-Mab production was 272  $\mu\text{g/ml}$ . The mini-Mab production level was the highest (320  $\mu\text{g/ml}$ ) at a glucose concentration of 0.1 %. The mini-Mab production appeared to be associated with the cell growth in the production medium because the final cell density for the medium with 0.1 % glucose was approximately 3 times higher than that for the medium without glucose. At a concentration of 1 % and when the medium was not changed from the growth medium to the production medium, there was no mini-Mab production. At a high glucose concentration, acetate may have been formed and inhibited the mini-Mab

production (Imanaka, et al., 1973). The glucose concentration at 0.1 % was kept for our further studies, as in the original protocol.

### 3. Optimal Times for the Cell Growth and for Mini-Mab Harvest

Optimal Time for the Completion of Cell Growth. At the end of the first stage, when the medium optical density (O.D.) becomes 0.9, cells are separated from the growth medium and transferred to the mini-Mab production medium. In order to accurately predict the time for the completion of the cell growth without frequently measuring the optical density of the growth medium, the growth of E4FX was observed with respect the time.

As can be seen in Figure 6, the lag phase lasted for hours. Then during the exponential growth phase, the doubling time of E4FX was approximately 30 minutes.

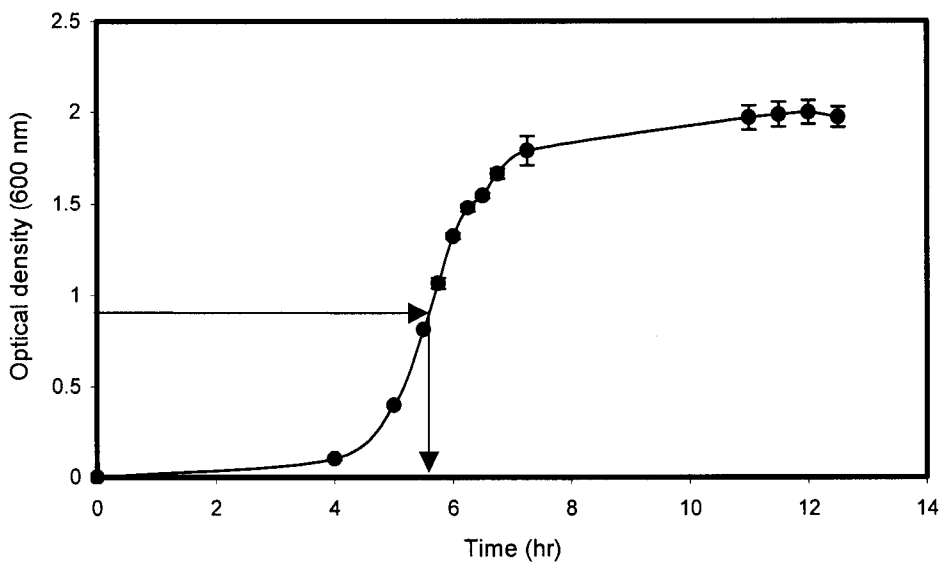


Figure 6. Cell growth curve of *E. coli* HB2151 E4FX. [Experimental conditions: 35 °C and 1% glucose]

The time to reach the O.D. of 0.9 after the cell inoculation was approximately 5 hours and 30 minutes with very little deviation.

Optimal Production Time. In the original protocol, the time for completing mini-Mab production after adding the inducer IPTG was 24 hours. To obtain the optimal time for the mini-Mab harvest, the concentration of the mini-Mab in the medium was measured in time (Figure 7). Over the initial five hours, very little mini-Mab was produced. Then from 5 to 15 hours, the mini-Mab production was almost linear with time and the production was complete at the time of 18 hours. After 18 hours, the amount of the mini-Mab decreased, showing possible mini-Mab degradation by protease in the medium. Therefore, 18 hours after adding IPTG, was selected as our optimal mini-Mab production time.

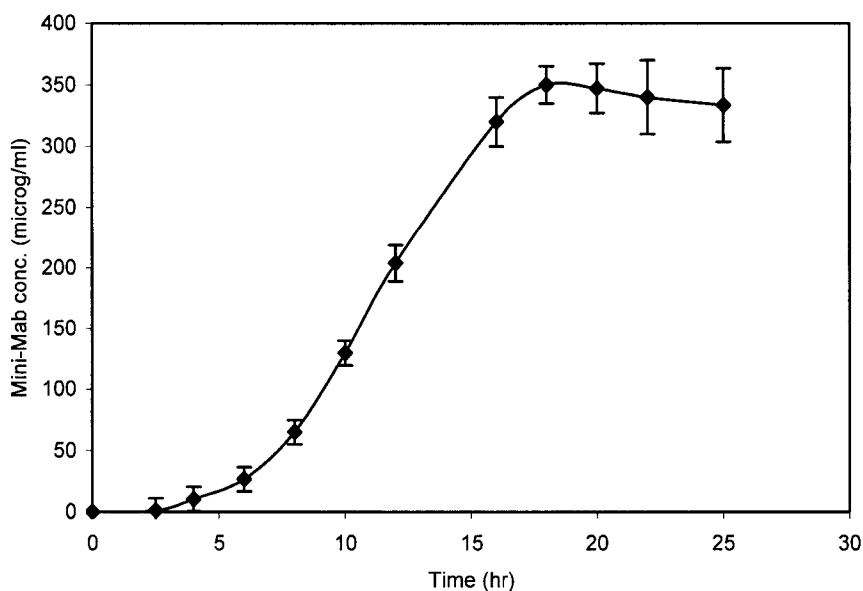


Figure 7. Mini-Mab production time after adding of IPTG. [Experimental condition: 30 °C, initial pH 7.0, and 0.1 % glucose]

#### 4. Effect of the Production Medium pH on the Mini-Mab Production

The pH in the growth medium at O.D. 0.9 is approximately 6, the initial pH of the mini-Mab production medium is 7, and the pH of the production medium after completing the mini-Mab production is 9. Since there are significant changes in the media pHs at the end of the cell growth, at the beginning and at the end of the mini-Mab production, the effect of initial pH on the mini-Mab production was investigated.

The initial pH of the production medium was adjusted at various pHs between 5 and 9 and the mini-Mab production was quantified (Figure 8). The final pH for the one starting at pH 5.0 was 5.3 and no mini-Mab production was observed. The final production media pHs of all other cases were between 8.4 and 8.8. The mini-Mab

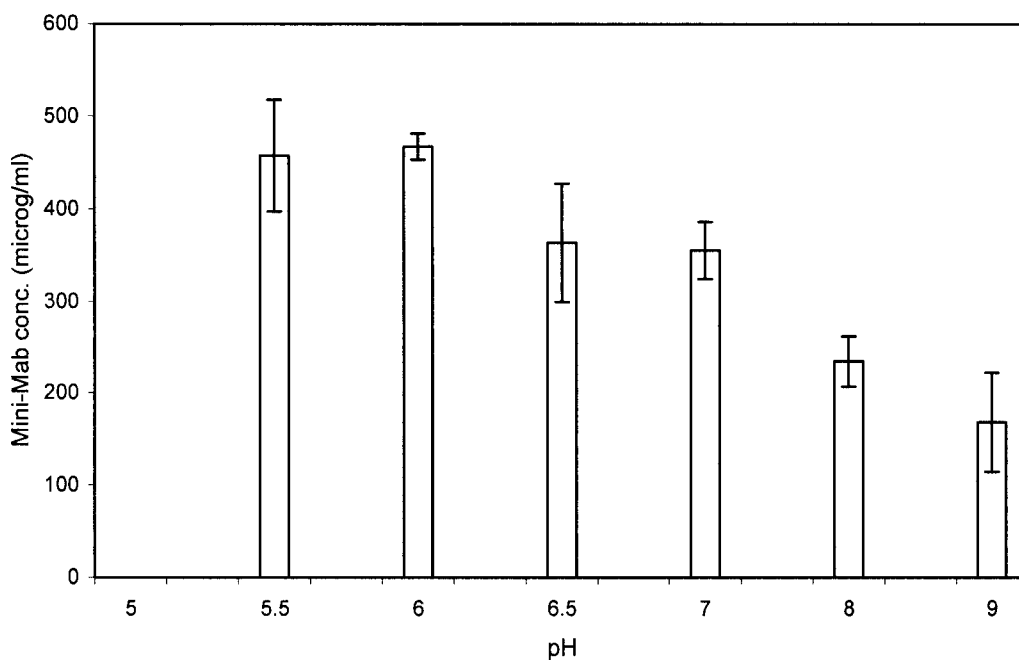


Figure 8. The effect of initial pH of the production media on the mini-Mab production. [Experimental condition: 18 hours of production time, 30 °C, and 0.1 % glucose]

production levels at the initial pH 5.5 and 6 were similar and the greatest. For ones at pH higher than 6, production decreased as pH increases. At the pH 9, the production is only 1/3 of that of the pH 5.5 or 6.0. The pH between 5.5 and 6.0 was decided to be the optimal initial pH for mini-Mab production.

5. Temperature

It was reported that the production of the mini-Mab produced by phage display method is affected by temperature (Kempf, et al., 2001). The effect of temperature on the mini-Mab production was, therefore, investigated in a temperature range of 23~35 °C (Figure 9).

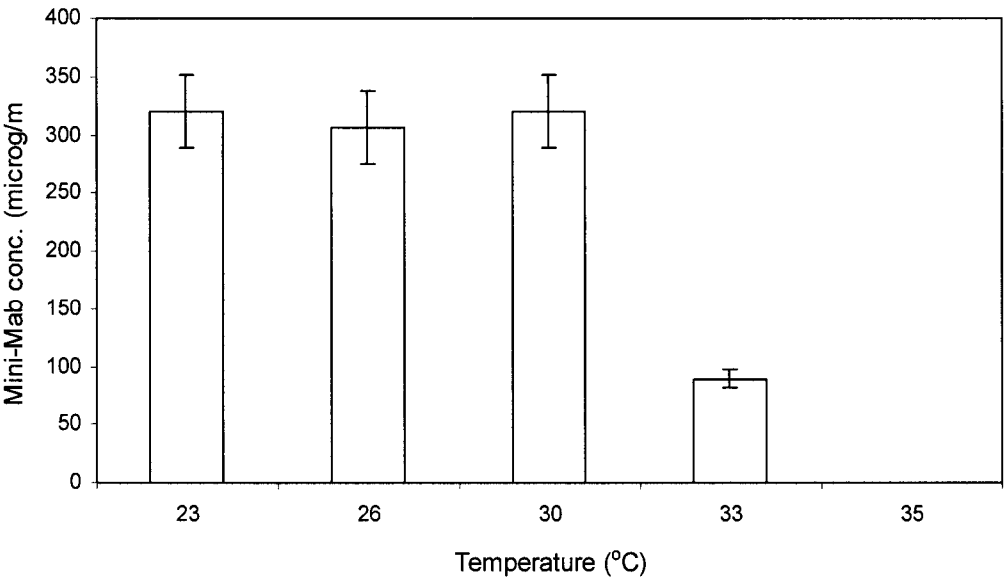


Figure 9. The effect of temperature on mini-Mab production. [Experimental condition: 18 hours of production time, initial pH 7.0, and 0.1 % glucose]

The production at temperature up to 30 °C showed a constant level of around 320 µg/ml. The production level at 33 °C was significantly low, and at 35 °C, the production was almost zero. The results show that the production is stable at a reasonable room temperature as long as the temperature is below 30 °C.

## 6. IPTG Concentration

The most expensive component of the production medium is the inducer, isopropyl β-D-thiogalactopyranoside (IPTG). The IPTG concentration used in the original protocol was 1 mM (Wu, et al., 1998). The study performed by Korah, et al. (2003b) showed that when IPTG was used at a level of 1 mM, its cost becomes 25 % of the total media cost. IPTG concentrations of 0, 0.05, 0.1, 1, and 2 mM were tested by

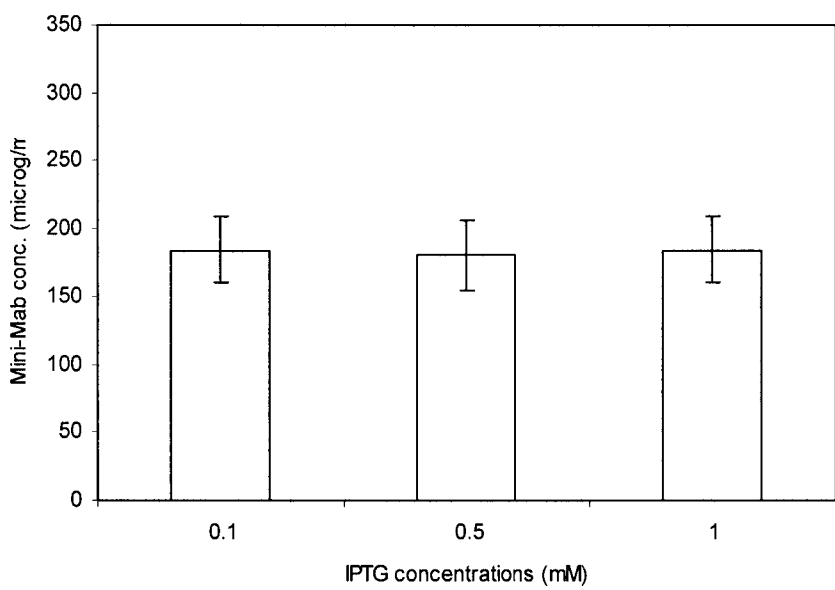


Figure 10. The effect of IPTG concentration on mini-Mab production.

[Experimental condition: 18 hours of production time, 30 °C, and 0.1 % glucose]



Korah, et al. (2003b). It was found that the production rate of mini-Mab was the greatest at 0.1 and 1 mM of IPTG.

Here, the mini-Mab production between at 0.1 and 1 mM of IPTG was investigated. The results (Figure 10) showed that the production levels of the mini-Mab at 0.1, 0.5, and 1 mM IPTG concentrations were almost the same. Therefore, the optimum concentration of IPTG was confirmed to be 0.1 mM.

### 7. Lactose as an Inducer

Kilikian, et al. (2000) reported that lactose may be used as an inducer for recombinant intracellular protein production. The amount of the chicken muscle Troponin C produced by the recombinant *E. coli* using lactose as an inducer (96 mg/g-dry cell weight) was similar to that using IPTG (110 mg/g-dry cell weight).

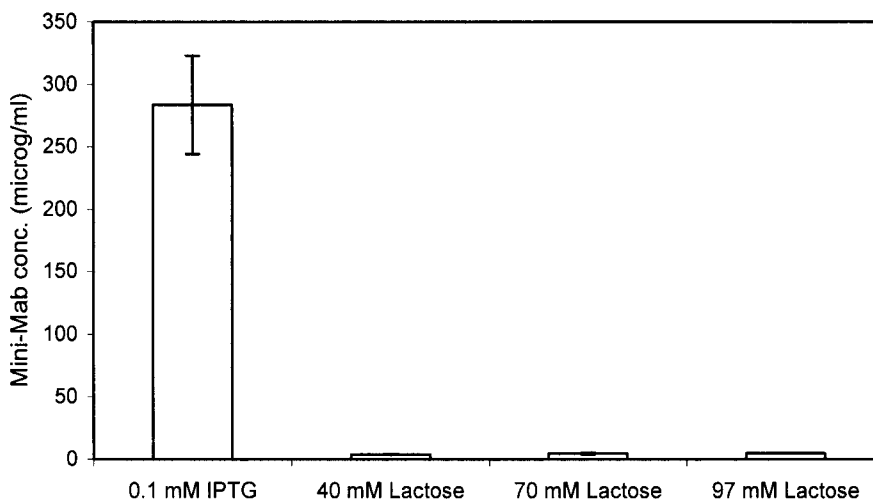


Figure 11. The effect of lactose as an inducer on mini-Mab production.

[Experimental condition: 18 hours of production time, 30 °C, and 0.1 % glucose]

Since lactose is approximately 1 % of the cost of IPTG, lactose at three different concentrations, 15 (40 mM), 25 (70 mM), and 35 g/L (97 mM) following the concentrations used by Kilikian, et al. (2000) were tested (Figure 11). Very little mini-Mab was produced at all three lactose concentrations and it was concluded that lactose was not proper as an inducer for the mini-Mab production.

#### 8. Mini-Mab Release from *E. coli* Cells

The mini-Mab developed by Dr. Michael Sierks at Arizona State University was designed to release mini-Mab outside of *E. coli* cells (Wu, et al., 1998a). However, the actual mini-Mab release status was not confirmed. Korah (2002) has previously tested the release of mini-Mabs in the *E. coli* cells by loosing cell membranes using ultrasonication and found that ultrasound denatured the mini-Mab, indicating that the mini-Mab is pressure sensitive.

Sucrose. There have been reports that a high level expression of mini-Mabs in *E. coli* often results in aggregation after their transport to the periplasm (Whitlow and Filpula, 1991; Kipriyanov, et al., 1994). Kipriyanov, et al. (1997) also reported that sucrose at 0.4 M helped the aggregated mini-Mab secrete outside of *E. coli* because sucrose is small enough to diffuse into the periplasmic space of *E. coli* but not metabolized. Therefore, the sucrose effect on the PC mini-Mab release was tested. The production medium was prepared with and without sucrose. The mini-Mab production was performed. Mini-Mab concentrations in the medium were measured after the completion of the mini-Mab production (Figure 12). The mini-Mab concentration in the medium with sucrose was 20 % lower than that without sucrose.

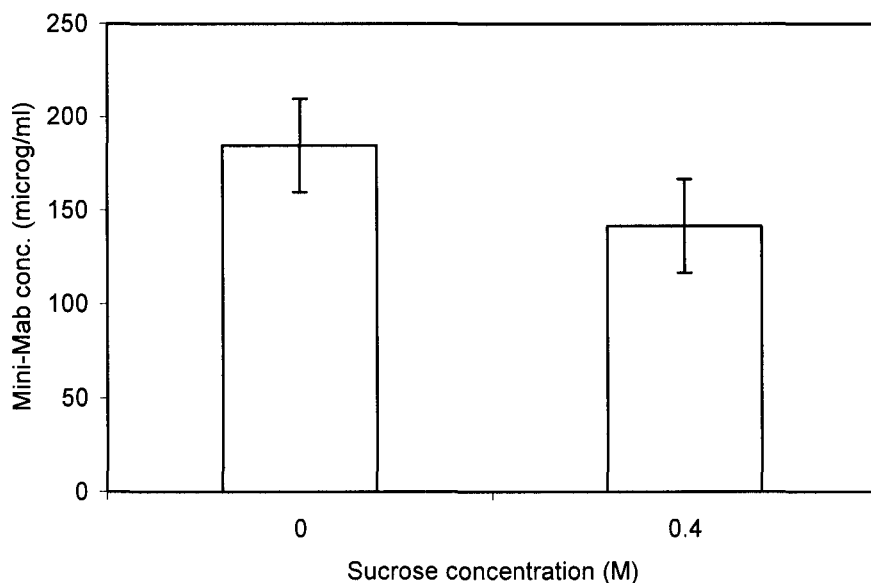


Figure 12. The effect of sucrose on the mini-Mab release. [Experimental condition: 18 hours of production time, 30 °C, and 0.1 % glucose]

Detergent and Membrane Reactive Cationic Peptides. There have been studies on the release of the recombinant proteins from the periplasm using non-ionic detergents and cationic peptides, such as, tetraethyleneglycolmonodecyl-ether, Triton X-100, and phospholipase A2 (Morbe, et al., 1997). The outer membrane of gram-negative bacteria is impermeable to macromolecules. Only some hydrophobic molecules, such as non-ionic detergents, may diffuse through the lipopolysaccharide (LPS), which is a major component of membranes (Nikaido, 1990). Cationic substances, such as cationic peptides, bind to the LPS containing membranes because of the polyanionic nature of the membrane, leading the membrane permeable (David, et al., 1996).

Therefore, in this study, two reagents, phospholipase A2 in PBS and Triton X-100 were tested in PBS buffer.

After 18 hours mini-Mab production, the cells were separated using the centrifuge at 4,000 rpm for 20 minutes, washed with PBS buffer, and re-suspended in 5 ml of PBS buffer. The permeabilizing agents were added to the cell-containing buffer at a final concentration of 0.4 µg/ml for phospholipase A2 and 2 % for Triton X-100. PBS buffer was used as a control.

After 40 minutes of reaction, the cells were separated by centrifugation at 4,000 rpm for 20 minutes, and the mini-Mab in the supernatant was quantified by ELISA (Table 2). In the PBS buffer (control), approximately 50 µg of mini-Mab was measured and in phospholipase A2 showed less mini-Mab release. When triton X-100 was used, no mini-Mab was detected by ELISA.

Therefore, it is concluded that most of the mini-Mab produced by *E. coli* is released outside of the cell or the reagents tested are not effective on releasing the mini-Mab from the cell.

Table 2. The effect of a membrane reactive cationic peptide (phospholipase A2) and a detergent (Triton X-100) on the mini-Mab release from the cell. [Experimental condition: a final concentration of 0.4 µg/ml for phospholipase A2 and 2% for Triton X-100]

	Total mini-Mab release detected by ELISA (µg)
PBS buffer (control)	51.2±2.1
Phospholipase A2	17.5±0.05
Triton-100	-

## 9. Effects of PMSF on Mini-Mab Production and Storage

In the original protocol of the PC mini-Mab production, 1 mM of PMSF was added in the production medium (Wu, et al., 1998). Mini-Mabs have been reported to be degraded by the proteases in the medium and a serine protease inhibitor PMSF has been frequently added in the production medium to inhibit the protease activity (Whitlow, et al., 1993). It was also reported that PMSF improved the antibody stability during storage at 4 °C (Brichta, et al., 2003). PMSF is, however, toxic in human body by inhibiting the serine protease (Bollen, et al., 1988). Here, the effects of PMSF on the mini-Mab production and on preventing the mini-Mab from being degraded during storage were investigated.

PMSF was added to the production medium at a concentration of 0, 0.1, or 1 mM and the production was preceded. At the end of the production, the amount of the mini-Mab in the supernatant was quantified by ELISA (Table 3). The mini-Mab production decreased with the increase in PMSF concentration. At the PMSF concentration of 1 mM, the production level was only 60 % of the one with no PMSF. When the mini-Mab production was studied per unit dry cell weight, the mini-Mab production levels for both with and without PMSF were approximately the same, i.e. 90 mg/g-dry cell weight.

Table 3. The effect of PMSF on the mini-Mab production. [Experimental condition: 0.1 mM of IPTG concentration, initial pH 6.0, and 18 hours of production time]

PMSF	Mini-Mab concentration (µg/ml)	Mini-Mab production per cell weight (mg/g)
No PMSF	560±35	90.0±16
0.1 mM	430±88	90.1±18
1 mM	350±74	90.0±19

However, the total cell mass for the medium with 1 mM of PMSF was approximately 40 % less than that without it, showing possible cell growth inhibition by PMSF.

The effect of PMSF on the mini-Mab degradation during the mini-Mab storage was also studied. The culture broth was divided into six portions: Two were stored in -70 °C without PMSF, with or without cells for 12 days; two were stored at 4 °C with 3 mM of PMSF, with or without cells for 9 days; the other two were stored at 4 °C without PMSF, with or without cells for 9 days. After the storage, the mini-Mab in the medium was quantified by ELISA (Table 4). When the culture broth was stored at -70 °C, the mini-Mab was kept well with no activity reduction for both with and without cells. At 4 °C, even with PMSF, there was significant degradation (> 22%). In the medium without PMSF, there was 10 ~ 16% more degradation than that with PMSF. When cells were present in the medium, the degradation was approximately 15 % more than without cells. From the study result, it is concluded that the best way of storing the mini-Mab produced medium is storing at -70 °C. If the medium has to be stored at 4 °C for some reason, the cells should be removed first and stored with PMSF.

Table 4. The effect of PMSF on the mini-Mab storage.

Storage temperature	Storage conditions	Mini-Mab concentration (µg/ml)
	Initial Concentration	351±35
- 70 °C (12 days)	w/o PMSF, w/ and w/o cells	351±16
4 °C (9 days)	w/ PMSF, w/ cells	211±15
	w/ PMSF, w/o cells	274±12
	w/o PMSF, w/ cells	153±28
	w/o PMSF, w/o cells	240±45

## B. MINI-MAB PURIFICATION

### 1. Mini-Mab Purification Using Protein A

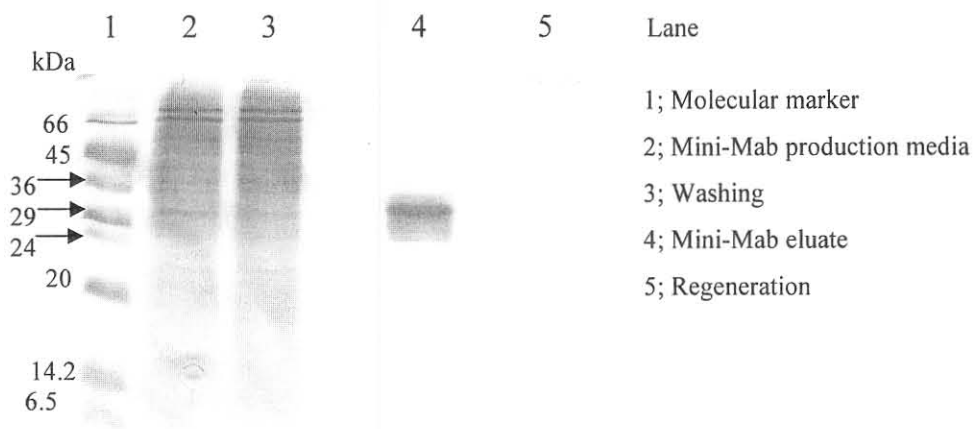
Protein A is a cell wall protein of *Staphylococcus aureus* and has affinity to the V<sub>H</sub> domain of a mini-Mab (Akerstrom, et al., 1994; Wilkinson, 2000). Korah (2002) previously reported 18 % of PC mini-Mab purification yield using the protein A immobilized CNBr-activate Sepharose<sup>TM</sup> at a elution buffer pH 3.0. For the matrix preparation, she immobilized 0.45 g of protein A for 1 ml of CNBr Sepharose<sup>TM</sup>. In this study, the mini-Mab was purified by a commercially available protein A immobilized CNBr-activated Sepharose<sup>TM</sup> CL-4B matrix (protein A Sepharose<sup>TM</sup>). The manufacturer's specification states that approximately 2 mg of protein A can be immobilized per 1 ml gel of the CNBr-activated Sepharose<sup>TM</sup> CL-4B. 100 ml of mini-Mab containing supernatant at a mini-Mab concentration of approximately 450 µg/ml was applied to a column containing 1 ml of protein A Sepharose<sup>TM</sup>. After the adsorption and washing steps, the glycine buffer of pH 3.0 (Korah, 2002) was applied to elute the adsorbed mini-Mab. The amount of mini-Mab in the eluate was quantified by ELISA. The purification yield [(mini-Mab mass in the eluate)/(mini-Mab mass in the applied supernatant) x 100] was 35 % (Table 5).

Table 5. Mini-Mab purification by Protein A Sepharose<sup>TM</sup>. [Experiment conditions: 1 ml of gel matrix and 0.015 cm/s of linear velocity]

Elution buffer	pH	Yield of mini-Mab purification (%)
Glycine	3	35±1.4
Citric acid	3	34±1

For the PC purification, the purified PC mini-Mab needs to be immobilized on the affinity gel matrices. The glycine buffer (linear structure formula:  $\text{NH}_2\text{-CH}_2\text{-COOH}$ ) used for the mini-Mab purification has a  $\text{NH}_2$  residue and the residue was found to compete with the mini-Mab during the immobilization of the mini-Mab on the matrix. Therefore, another buffer that does not interfere the immobilization efficiency was explored. Citric acid [ $\text{CH}_2(\text{COOH})\text{-COH}(\text{COOH})\text{-CH}_2(\text{COOH})$ ] buffer was investigated at the elution pH of 3.0. For the purpose of mini-Mab purification, three different pHs of the elution were tested. Table 5 shows the purification yields of mini-Mab eluates by glycine and citric acid buffers. The yield of the mini-Mab eluted by citric acid buffer was 34 %, which is similar to that by glycine buffer. For further studies, citric acid was used for the mini-Mab elution.

## 2. Electrophoresis of Mini-Mab Purified from the Production Supernatant.



**Figure 13.** Electrophoresis of the samples of the mini-Mab purification from the production supernatant on Protein A Sepharose<sup>TM</sup>.



Various fractions from the mini-Mab purification process were analyzed by gel electrophoresis under non-reduced conditions (Figure 13). The result showed a single band around 30 kDa of the molecular weight, indicating that the eluate contains only the mini-Mab.

### C. PC PURIFICATION

#### 1. Cross reactivity of mini-Mab against PC and PC homologues

Using the purified mini-Mab, the cross reactivity against PC and PC homologues was investigated by ELISA. The mini-Mab at a concentration of 2  $\mu\text{g/ml}$  was applied on each well of ELISA plate and each 1  $\mu\text{g/ml}$  of PC and PC homologues was adsorbed on the mini-Mab and 1  $\mu\text{g/ml}$  of each monoclonal antibody against the PC and PC homologues were applied to the adsorbed PC and PC homologues.

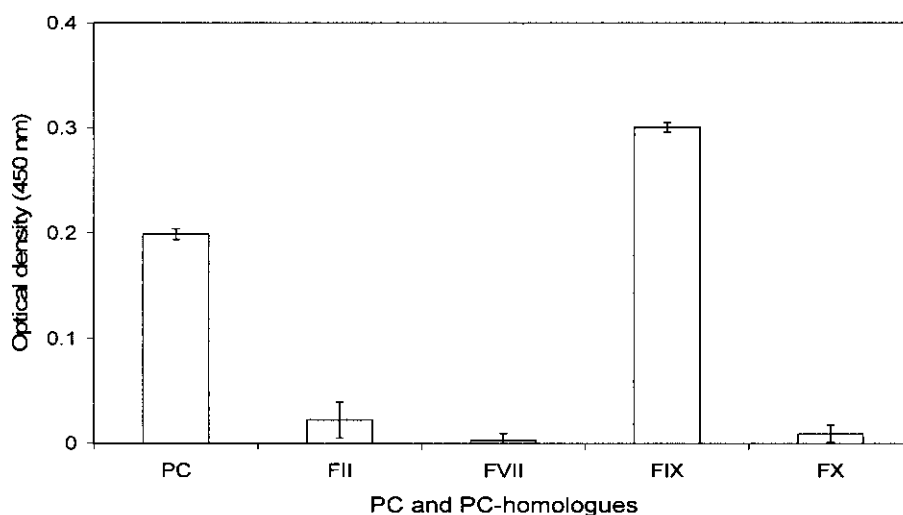
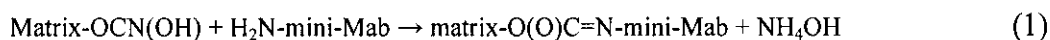


Figure 14. Cross reactivity of mini-Mab against PC and PC homologues.

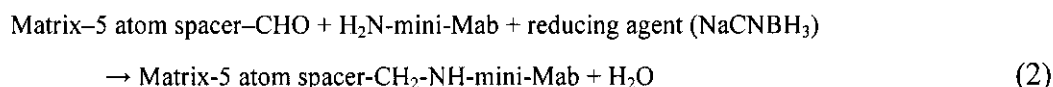
The optical density (O.D.) of the developed color in ELISA wells were measured at a wavelength of 450 nm (Figure 14). The optical densities generated by factors II, VII, and X were minimal, indicating that the mini-Mab has little cross reactivity against the factors II, VII, and X. However, factor IX showed 1.5 times higher O.D. of PC.

## 2. Selection of Gel Matrices

When a biomolecule is covalently immobilized on matrix, the length of the spacer arm on the matrix may affect the activity of the immobilized biomolecule (Kang, et al., 1992). To study the effect of spacer arm length on PC purification performance of gel matrices, commercially available, four gel matrices with various lengths of the spacer arm were selected. They are CNBr Sepharose<sup>TM</sup>, Actigel<sup>TM</sup>, NHS Sepharose<sup>TM</sup>, and Epoxy-activated<sup>TM</sup>. CNBr Sepharose<sup>TM</sup> is 4 % agarose and reacts with amine residues of ligands for immobilization via one oxygen atom spacer (Eq. 1; Amersham Biosciences).

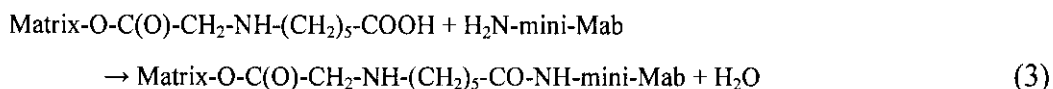


Actigel<sup>TM</sup> is 4 % cross-linked agarose and its monoaldehyde group reacts with amine residues of proteins, via 5-atom spacers (Eq. 2; Sterogene Bioseparations).

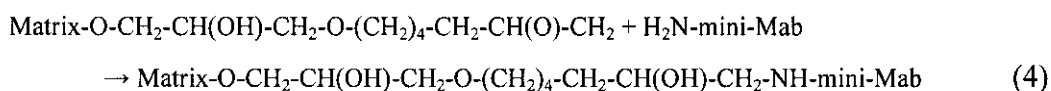


NHS Sepharose<sup>TM</sup> is 4 % cross-linked agarose and has 6-amino-hexanoic acid forming 10-atom spacers of oxygen and carbon. The terminal carboxyl group is activated by esterification with N-hydroxysuccinimide (NHS) and the amino group of the mini-Mab

couples to this active ester to form a stable amide linkage (Eq. 3; van Sommeren, et al., 1993).



Epoxy Activated matrix is 4 % cross-linked agarose and its epoxy group binds to amines of the ligands via 12-atom spacer of oxygen and carbon (Eq. 4).



Korah et al. (2003a) has tested CNBr Sepharose<sup>TM</sup> and Actigel<sup>TM</sup> for PC purification using pure PC. Actigel<sup>TM</sup> showed a PC purification efficiency higher (10 %) than CNBr Sepharose<sup>TM</sup> (6 %), indicating that the matrix with a longer spacer arms may keep the 3-D structure of the immobilized mini-Mab better.

2 mg of the mini-Mab was reacted with 1 ml of each matrix. The mini-Mab immobilization efficiencies of the CNBr Sepharose<sup>TM</sup>, Actigel<sup>TM</sup>, NHS Sepharose<sup>TM</sup>, and Epoxy Activated<sup>TM</sup> matrices were approximately 85, 85, 95, and 70 %, respectively, showing NHS Sepharose with the highest (Table 6). Each matrix was packed in a column, the PC performance was tested using 20 µg of pure PC, and the PC in the eluate was quantified by ELISA (Table 6). The purification efficiency was presented as a normalized PC amount per 1 mg of the immobilized mini-Mab on the matrices. CNBr Sepharose<sup>TM</sup> showed the worst purification efficiency probably because the mini-Mab bound to the matrix via a very short spacer, losing its original three dimensional conformations (Kang, et al., 1992). Actigel<sup>TM</sup> showed a reasonable performance and NHS Sepharose<sup>TM</sup> has shown the best performance for the PC purification (54 %).

Table 6. PC purification performance by mini-Mab immobilized matrices.

	CNBr Sephacrose™*1	Actigel™	NHS Sephacrose™	Epoxy Activated™
Length of the spacer (number of atoms)	1	5	10	12
Mini-Mab immobilization efficiency*2( $\epsilon_1$ )	0.85	0.85±0.03	0.95±0.02	0.7
PC purification yield*3 ( $\epsilon_2$ )	0.06±0.01	0.48±0.18	0.54±0.11	0.30±0.07
Normalized PC purification yield ( $\mu\text{g-PC/mg-mini-Mab}$ )	1.1±0.3	5.6±2.1	5.7±1.8	4.9±1.0
Matrix productivity ( $\epsilon_1 \times \epsilon_2$ )	0.05	0.41	0.51	0.21

\*1: The study using CNBr Sepharose was performed by Korah (2002).

\*2 ( $\epsilon_1$ ) and \*3 ( $\epsilon_2$ ): The related information was reported by Kang, et al. (1992).

The normalized PC purification yield of NHS Sepharose™ showed that 10-atom spacer arms have kept the activity of the immobilized mini-Mab well. Epoxy Activated™ has spacers longer than NHS Sepharose™ but showed lower immobilization efficiency than other matrices and also the immobilized mini-Mab on the gel showed a purification performance inferior to that of Actigel™ or NHS Sepharose™. Kang, et al. (1992) evaluated the matrix performance by the coupling efficiency of ligand ( $\epsilon_1$ ), the product adsorption efficiency of ligand ( $\epsilon_2$ ), and the specific activity of the eluted product ( $\epsilon_3$ ). Here, the mini-Mab immobilization efficiency ( $\epsilon_1$ ) and PC purification yield ( $\epsilon_2$ ) of the matrices were used to study the PC purification performance, assuming that the activities of the purified PC by the matrices are the same. The NHS Sepharose™ showed

the best PC purification performance and, therefore, NHS Sepharose<sup>TM</sup> was selected for the further PC purification studies.

### 3. PC Purification from the PC Homologues Mixture

As previously stated, PC sources, plasma and Cohn Fraction IV-1 include many proteins homologues to PC and these homologous proteins are coagulants. To study the optimal amounts of the mini-Mab immobilized on NHS Sepharose<sup>TM</sup> for PC purification, 2, 4, and 6 mg of the purified mini-Mab were immobilized on 1 ml of the NHS Sepharose<sup>TM</sup> matrix each. A mixture of PC and PC homologous coagulant factors was used as a source material and PC purification performance of each matrix was studied.

Commercially available PC homologous blood clotting factors were mixed with PC following the physiological ratio in plasma, i.e., PC, 4; factor II, 90; factor VII, 0.5; factor IX, 4; and factor X, 6.4 µg/ml. The actual amounts of proteins were: PC, 20; factor II, 450; factor VII, 2.5; factor IX, 20; and factor X, 32 µg, in the final volume of 500 µl. PC and PC-homologues in the fraction at various chromatography steps were quantified by ELISA (Table 7).

When 2 mg of the mini-Mab was used, approximately 85 % of PC was washed away, showing much less adsorption compared with the case with PC only. Nevertheless, more than 95 % of PC homologues were also washed away. During the elution process, 14 % of PC yield was recovered, much less than when the pure PC was used as the source material (54%). For the PC homologues, less than 5 % of PC homologues remained in the eluate. However, the amount of factor II in the eluate was greater than that of PC, because of its large amount in the source material (more than 20

times greater than PC). Factor IX, which showed a high affinity to the mini-Mab for the ELISA did not remain in the column.

For the matrix with 4 mg of the mini-Mab, 73 % of PC was washed away, 10 % less than that with 2 mg. As in the matrix with 2 mg of mini-Mab, more than 95 % of PC homologues were washed away. The amount of factor II in the eluate was less than that of PC. During elution, yield of 26 % was obtained.

Table 7. PC purification efficiency when 2 mg, 4 mg, or 6 mg of the mini-Mab was immobilized in 1 ml of the matrix. The source material was the mixture of PC and four PC homologues. [Experiment conditions: 0.015 cm/s of linear velocity, and 10 minutes of reaction time]

Purification steps	Amount of immobilized mini-Mab (mg/ml-matrix)		Amount of factors				
			PC	FII	FVII	FIX	FX
Washing	2	µg (%)	17.1±1 (85±3)	441±1 (98±0.3)	2.5±0.1 (99±1)	19.6±0.1 (98±0.1)	30.2±0.6 (95±2)
	4	µg (%)	14.6±1 (73±9)	441±5 (98±1)	2.4±0.1 (97±2)	19.7±0.2 (98±1)	30.7±0.1 (96±0.1)
	6	µg (%)	14.6±1 (73±3)	442±5 (98±1)	2.4±0.1 (95±3)	19.6±0.3 (98±1)	31.2±0.9 (97±3)
Elution	2	µg (%)	2.7±1 (14±3)	5±1 (1±0.2)	0 (0)	0.1±0.1 (0.3±0.2)	1.1±1.2 (3±4)
	4	µg (%)	5.2±1 (26±4)	5±5 (1±1)	0.1±0.1 (2±3)	0.1±0.1 (0.7±0.7)	0.5±0.4 (2±1)
	6	µg (%)	5.2±1 (26±3)	7±4 (2±1)	0.1±0.1 (4±5)	0.2±0.2 (1.1±1.0)	0.7±0.8 (2±2)

For the NHS matrix with 6 mg of the mini-Mab, 73 % of PC was washed away, which is similar to that with 4 mg of mini-Mab, and more than 95 % of PC homologues were washed away. The PC purification yield of 26 %, similar to that of the matrix immobilized with 4 mg of mini-Mab, was obtained. The amount of factor II in the eluate was greater than that of PC. The amounts of other PC homologues in the eluate (factors II, IX, and X) were slightly more than those in the case of the 4 mg mini-Mab.

This study result shows that the NHS Sepharose matrix with 4 mg mini-Mab showed the best performance and was used for further studies for PC purification from Cohn Fraction IV-1. The result also shows that PC purification yield decreases significantly when there are other PC homologous proteins exist in the source material and the amount of factor II in the eluate is similar to or slightly more than that of PC.

#### 4. Purification of PC from Cohn Fraction IV-1

Quantification of PC and PC Homologues. It has been reported that 1 kg of Cohn Fraction IV-1 paste had approximately 100 mg of PC (Wu, et al., 1998). The concentrations of the PC homologues in Cohn Fraction IV-1, however, have not been quantified. Here, PC and the PC homologues in the Cohn Fraction IV-1 were quantified using ELISA.

Following Wu's protocol (2000b), approximately 22 g of Cohn Fraction IV-1 paste was dissolved in 440 ml of sodium citrate buffer (approximately 20 times dilution ratio; pH 6.0) and placed for 5 hours, at 4 °C, with stirring at 100 rpm. After the centrifugation of the paste solution at 4000 rpm for 20 minutes, PC and the homologues in the supernatant were quantified using ELISA (Table 8).

Table 8. Amounts of PC and PC homologues in Cohn Fraction IV-1 paste.

	PC	FII	FVII	FIX	FX	HSA
Human plasma ( $\mu\text{g/ml}$ )	4 [1]*	90 [23]	0.5 [0.13]	4 [1]	6.4 [1.6]	40000 [10000]
Supernatant of Cohn Fraction IV-1 (mg/kg-paste)	103.9 $\pm$ 3.4 [1]	1201 $\pm$ 178 [12]	29.3 $\pm$ 5.7 [0.3]	13.5 $\pm$ 3.1 [0.13]	269 $\pm$ 18 [2.6]	24812 $\pm$ 5720 [239]
After the filtration of Cohn Fraction IV-1 (mg/kg-paste)	95.5 $\pm$ 2.3 [1]	1171 $\pm$ 15 [12]	20 $\pm$ 1.2 [0.2]	9.2 $\pm$ 0.2 [0.1]	264 $\pm$ 9 [2.8]	22777 $\pm$ 4410 [239]

[ ]\*: the numbers in brackets are showing the ratio of the amount of factors to PC amount.

The amount of PC in the supernatant was determined to be approximately 100 mg/kg-paste and the ratio of the amount of factors VII and IX to that of PC's was much less. The amounts of factor X was approximately 3 times more than that of PC. The amounts of factor II was shown to be approximately 1200 mg/kg-paste. For human serum albumin (HSA), which is the most abundant protein in plasma, the amount ratio to that of PC was forty times less than that in plasma.

The supernatant was then filtered using polyethersulfone filters (0.8, 0.45, and 0.2  $\mu\text{m}$  of pore sizes, respectively) to remove particulate before applying it to mini-Mab immobilized gel matrix for the PC purification. After filtration, approximately, 8 % less amount (96 mg/kg-paste) of PC and 2~32 % less homologues were found in the filtrate (factor II; 1171, factor VII; 20, factor IX; 9.2, and factor X; 264 mg/kg-paste).

Effect of Adsorption pH on PC Purification Performance. The usual pH used for either plasma or in Cohn Fraction IV-1 is 6.0, to reduce the activity of protease present in



plasma (Amaral, et al., 2003; Vazquez-Lopez, et al., 1999). ELISA was carried out to study the affinity of the mini-Mab to PC at pH 6 and 7. The mini-Mab at pH 7 showed the affinity to PC 30 ~ 40 % higher than that in pH 6 (data not shown). Next, the effect of pH on the performance of the mini-Mab immobilized, PC affinity chromatography was studied. The pH of the source material was also adjusted to either pH 6 or 7. 4 ml of the Cohn Fraction IV-1 filtrate (total PC amount, 15 µg) was applied to the mini-Mab immobilized NHS matrix and the PC purification was performed. The amount of PC at various purification stages were determined by ELISA (Table 9).

Table 9. Amount of PC and PC homologues in various fractions during purification process from Cohn Fraction IV-1 at pHs of 6.0 and 7.0. The amount of the factors in washing and elution. [Experiment conditions: 1 ml of gel matrix, 0.015 cm/s of linear velocity, and 10 minutes of reaction time]

Purification steps	pH		Amount of factors				
			PC	FII	FVII	FIX	FX
Washing	6	µg (%)	11±2 (73±8)	464±209 (93±7)	1.2±0.5 (98±1)	1.3±0.4 (86±9)	2.7±0.1 (96±5)
	7	µg (%)	11±2 (73±17)	475±180 (96±1)	1.2±0.5 (99±1)	1.3±0.5 (86±14)	4±2.2 (94±2)
Elution	6	µg (%)	2.4±0.3 (16±1)	1.2±0.1 (0.3±0.1)	0 (0)	0.003±0.001 (0.3±0.1)	0.01± 0.003 (0.4±0.1)
	7	µg (%)	1.7±0.1 (11±1)	0.9±0.1 (0.2±0.1)	0 (0)	0 (0)	0.003±0.003 (0.1±0.1)

In the washing step, 73 % of PC was washed away for both pHs with the similar percentage of washing for PC homologues. For factor II, 93 and 96 % were washed away for pH 6 and 7, respectively. Approximately 98 and 95 % of factors VII and X were washed away for both pHs, respectively. For factor IX, approximately 86 %, less than the other factors, was washed away.

In the eluate, the amount of factor II was found to be half of the actual PC amount. For the other factors, less than 0.4 % was found in the eluate for both pHs. The PC in the eluate quantified by ELISA was shown to be 16 and 11 % at pHs of 6 and 7 respectively, showing approximately 5 % higher yield with pH 6, and possible PC degradation at pH 7.0. The yield of 16 % at pH 6.0 from the Cohn Fraction IV-1 is also almost a half of the yield from PC homologues mixture (26 %).

Effect of HSA in the source material on PC Purification Performance. As previously shown, there is a reduction in the purification yield for the sample of the mixture of PC and PC homologues compared with the sample with PC only. In the presence of large quantity of other molecules, PC molecules do not get to be transported to the mini-Mab well. Although human serum albumin (HSA) in Cohn Fraction IV-1 is only 1/30 of that in plasma, the amount is still 240 times greater than that of PC. Therefore, it was speculated that this large amount of HSA may hinder the diffusion of PC molecules. Therefore, the effect of the presence of large amount of HSA in the source material on PC purification was systematically studied. Sample composed of PC and PC homologues at a physiological concentration ratio in plasma (PC, 20; factor II, 450; factor VII, 2.5; factor IX, 20; and factor X, 32  $\mu$ g) with or without HSA (0.2 g) was applied to the mini-Mab immunoaffinity chromatography column and the purification

process was performed. Each factor in washing and elution steps was quantified by ELISA (Table 10).

In the washing step, for the sample without HSA, approximately 75 % of PC was washed away and for the samples with HSA, 88 %. For factor VII, 87 and 82 % were removed for the samples without and with HSA, respectively. For other factors, more than 96 % was removed.

In the elution, the PC purification yields were shown to be 12 and 25 % for the samples with and without HSA, respectively, showing that, with HSA in the source, the yield is reduced by approximately 13 %. The results show that the large quantity of HSA in the source material interferes the PC purification yield.

Table 10. Effect of HSA on PC purification. The amount of the factors in washing and elution steps (a) without and (b) with HSA. [Experiment conditions: 1 ml of gel matrix, 0.015 cm/s of linear velocity, and 10 minutes of reaction time]

Purification steps			Amount of factors					
			PC	FII	FVII	FIX	FX	HSA
Washing	(a)	$\mu\text{g}$ (%)	15 $\pm$ 1.4 (75 $\pm$ 7)	445 $\pm$ 0.6 (99 $\pm$ 0.1)	1.7 $\pm$ 1.1 (87 $\pm$ 13)	19.5 $\pm$ 0.2 (97 $\pm$ 2)	31 $\pm$ 0.1 (98 $\pm$ 0.2)	0 (0)
	(b)	$\mu\text{g}$ (%)	18 $\pm$ 0.4 (88 $\pm$ 2)	448 $\pm$ 0.5 (99.6 $\pm$ 0.1)	2.1 $\pm$ 0.1 (82 $\pm$ 4)	19.5 $\pm$ 0.4 (97 $\pm$ 2)	31 $\pm$ 0.4 (96 $\pm$ 4)	198934 $\pm$ 1323 (99.5 $\pm$ 0.6)
Elution,	(a)	$\mu\text{g}$ (%)	4.9 $\pm$ 1.3 (25 $\pm$ 7)	2.1 $\pm$ 0.6 (0.5 $\pm$ 0.1)	0.2 $\pm$ 0.3 (9 $\pm$ 1)	0.2 $\pm$ 0.1 (1.3 $\pm$ 0.4)	0.3 $\pm$ 0.1 (0.9 $\pm$ 0.3)	0 (0)
	(b)	$\mu\text{g}$ (%)	2 $\pm$ 0.3 (12 $\pm$ 2)	0.6 $\pm$ 0.1 (0.1 $\pm$ 0.01)	0.5 $\pm$ 0.1 (17 $\pm$ 4)	0.02 $\pm$ 0.01 (0.2 $\pm$ 0.1)	0.2 $\pm$ 0.2 (0.6 $\pm$ 0.5)	40.9 $\pm$ 23 (0.02 $\pm$ 0.01)

Effect of Salt on PC Purification. Since large amount of HSA in the source material affects PC purification yield significantly, it was also speculated that HSA molecules get adsorbed to the gel matrix non-specifically and prevent PC molecules from reacting with their mini-Mabs. The concentration of NaCl in washing buffer in the original protocol was 0.08 M and the washing buffer with 0.5 M of NaCl was tested. Cohn Fraction IV-1 with the PC amount ranging 15 ~ 20 µg was applied to the column and the PC purification was performed using the washing buffer at 0.5 M NaCl. The PC and PC homologues in washing and elution steps were quantified using ELISA (Table 11).

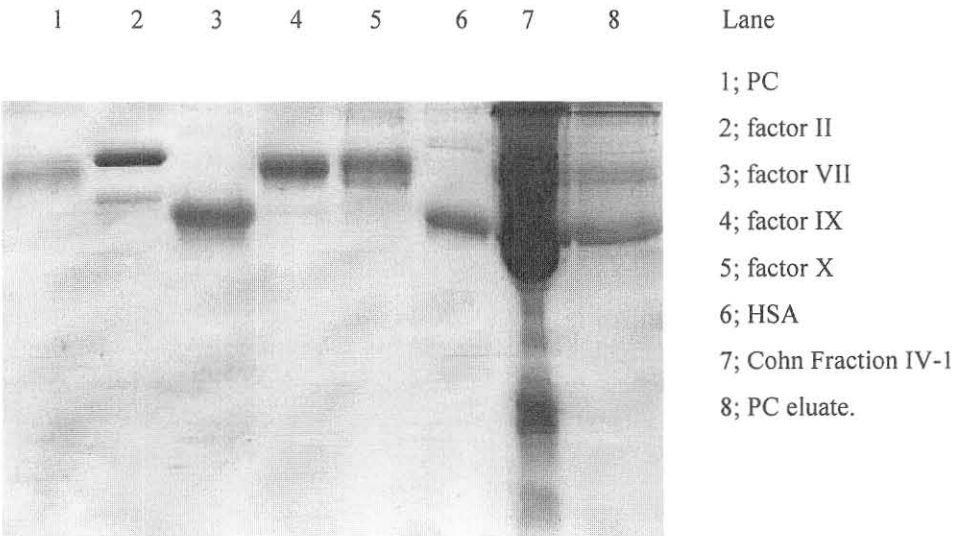
Table 11. Effect of the salt concentration in washing buffer on the purification of PC from Cohn Fraction IV-1. The amount of the factors in washing and elution steps when the washing buffer contains (a) with 0.08 and (b) 0.5 M NaCl. [Experiment conditions: 1 ml of gel matrix, 0.015 cm/s of linear velocity, and 10 minutes of reaction time]

Purification steps			Amount of factors					
			PC	FII	FVII	FIX	FX	HSA
Washing	(a)	µg (%)	11±0.4 (77±3)	533±90 (98±0.5)	4±4 (97±2)	1.2±0.5 (98±0.1)	6.2±2.7 (98±1)	5737±19 (99±1)
	(b)	µg (%)	18±2.3 (97±3)	618±21 (97±4)	3±1 (89±8)	1.0±0.4 (99.5±0.7)	7.5±0.6 (99±1)	6725±35 (99.8±0.1)
Elution	(a)	µg (%)	2±0.1 (15±0.4)	1.3±0.1 (0.2±0.1)	0 (0)	0 (0)	0.1±0.01 (1.6±0.1)	4±3.1 (0.1±0.01)
	(b)	µg (%)	0.1±0.1 (0.3±0.1)	0.7±0.2 (0.1±0.01)	0.5±0.5 (11±2)	0 (0)	0.02±0.01 (0.3±0.1)	15±0.3 (0.1±0.01)

During the washing step, for the washing buffer with 0.08 M NaCl, 77 % of PC was washed away, while 97 % of PC was removed for the buffer with 0.5 M NaCl. For other factors, more than 97 % were removed during the washing step both cases, but factor VII showed less amount of washing for using the buffer with 0.5 M of NaCl. In the elution, the PC purification yields were 15 and 0.3 % when the washing buffer had 0.08 M and 0.5 M of NaCl, respectively, showing that high salt concentration affects the PC adsorption to the mini-Mab. For other factors, less than 2 % remained for both cases except the factor VII remained more with 0.5 M NaCl.

5. Electrophoresis of Eluate of the PC Chromatography using Cohn Fraction IV-1

Electrophoresis was performed for commercially available PC, PC homologous factors, human serum albumin, and the fractions from the PC purification process from the Cohn Fraction IV-1 under non-reduced condition (Figure 15).



**Figure 15.** Electrophoresis of the samples from PC purification from Cohn Fraction IV-1 using the mini-Mab immobilized NHS Sepharose™.

The result showed that the mini-Mab PC affinity chromatography removed the proteins with molecular weights lower than PC. There are a single band around the molecular weight of PC (PC, factor II and human serum albumin, based on ELISA result). Two bands at the molecular weights greater than PC were also found in the eluate.

#### 6. Removal of HSA by DEAE Ion Exchange Chromatography

The isoelectric point (pI) of PC is 4.4~4.8 and the pI of HSA is 5.7 (Bordbar, et al., 2004). From the fact that a low PC purification yield occurs with the source material with high HSA concentration and there is a difference in pI between PC and HSA, ion exchange chromatography was considered to remove HSA before the mini-Mab affinity chromatography step.

Approximately 1 ml of the DEAE Sepharose<sup>TM</sup> Fast Flow matrix was packed in a column of the diameter at 0.7 cm. Then 100 ml of a filtered Cohn Fraction IV-1 solution was applied to the column and ion exchange chromatography was performed using a pH of 6.0 for adsorption/washing. PC, PC homologues, and HSA in washing and elution steps were quantified by ELISA (Table 12).

The yield of PC was approximately 65 % and 10 ~ 20 % of factors II, FIX, and FX were removed. 95 % of factor VII was removed. For the HSA, more than 99 % was washed away. The concentration of PC and PC homologues in the eluate became 10 times higher than that in the source, except the factor VII.

Table 12. The amount of PC, PC homologues, and HSA in the DEAE eluate of Cohn Fraction IV-1. [Experiment conditions: 1 ml of gel matrix, 0.004 cm/s of linear velocity, and 4 °C]

		PC	FII	FVII	FIX	FX	HSA
Amounts in the source	µg	644	10428	57	43	462	58857
Washing,	µg (%)	214 (33.3)	787 (7.5)	53 (94.4)	5.5 (13)	31 (6.7)	58723 (99.8)
Elution	µg (%)	420 (65.3)	8712 (83.6)	3 (5.3)	36.2 (85)	420.4 (90.9)	40.1 (0.07)
Conc. in the source	µg/ml	6.4	10.42	0.57	0.42	4.6	590
Conc. in the eluate	µg/ml	39	800	0.29	3	39	4

In summary, DEAE anion exchange chromatography removed most of the HSA and, PC and factors II, IX, and X were concentrated.

## 7. PC Purification from the Eluate of DEAE Chromatography Using the NHS Matrix

The affinity purification of PC from the eluate of DEAE chromatography of Cohn Fraction IV-1 was performed using the mini-Mab immobilized NHS matrix. The amount of PC of the DEAE eluate was approximately 20 µg and the amounts of HSA in the eluate was approximately 4 µg. PC, PC homologues, and HSA in elution step were quantified using ELISA (Table 13).

Approximately 25 % of PC purification yield was obtained, 9 % higher than that without the ion-exchange process (16 %). This result confirms that the large amount of HSA in the source material affects the adsorption of PC to the mini-Mab on the matrix.

Table 13. PC purification from the DEAE eluate of Cohn Fraction IV-1 using mini-Mab immobilized NHS matrix. [Experiment conditions: 1 ml of gel matrix, 0.015 cm/s of linear velocity, and 10 minutes of reaction time]

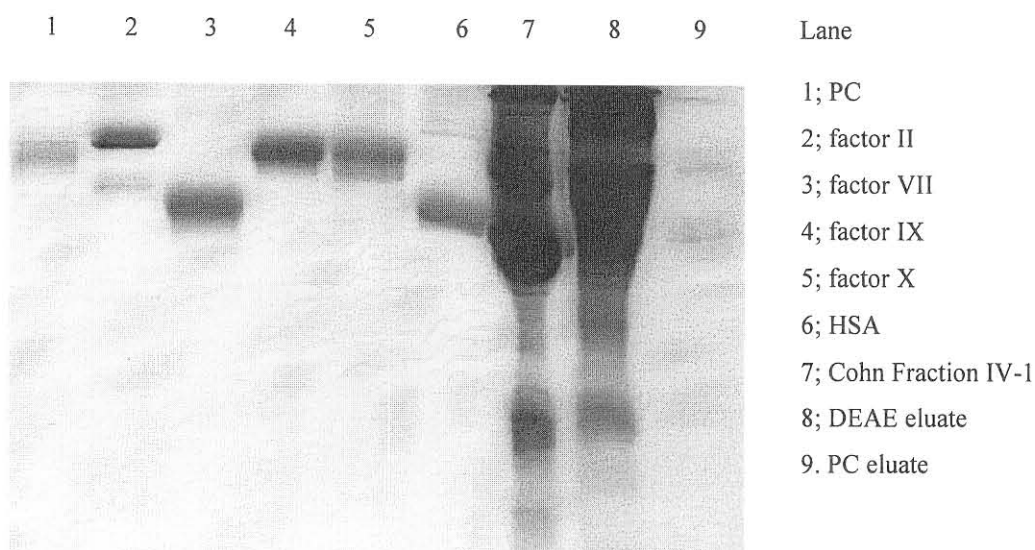
		PC	FII	FVII	FIX	FX	HSA
Amount in the source	μg	20	173±7	0.2±0.1	1.5±0.1	24±4	4±1.4
Amount in the eluate	μg (%)	4.9±0.1 (25±0.3)	2.5±1.4 (1.5±0.9)	0.1±0.1 (18±25)	0 (0)	0.1±0.02 (0.2±0.1)	0.3±0.01 (10±4)

The overall PC purification yield from Cohn Fraction IV-1 is combined value of the yield from DEAE anion exchange chromatography from Cohn Fraction IV-1 (65 %) and the yield (25 %) by mini-Mab immobilized NHS matrix using the eluate. It is approximately 16 %, and is similar to the PC purification yield from Cohn Fraction IV-1 without the ion-exchange process. For the other factors, the overall yields from Cohn Fraction IV-1 were similar to the values of the combined yields from both DEAE anion exchange chromatography from Cohn Fraction IV-1 and the purification from the eluate.

#### 8. Electrophoresis of Eluate of the PC Affinity Chromatography Using DEAE Eluate

Electrophoresis was performed for the eluate of the mini-Mab immunoaffinity using the eluate of DEAE chromatography, under a non-reduced condition (Figure 16). The PC eluate showed that there is a band around 60,000 Da, possibly for PC, factor II, and HSA. Two bands are shown at molecular weights greater than PC.





**Figure 16.** Electrophoresis of the eluate of PC purification using the mini-Mab immobilized NHS Sepharose<sup>TM</sup> with the source of the DEAE eluate.

#### D. ECONOMIC ANALYSIS OF PC PURIFICATION BY MINI-MAB

A preliminary economic analysis of PC purification using the mini-Mab was performed by Korah, et al. (2003). The specific production rate of mini-Mab was evaluated to be 500 times faster than that of Mab's. With pure mini-Mab, not the supernatant from the reactor broth, the yield of Mab purification (70 %) was evaluated 1.75 times greater than mini-Mab's (40 %) based on mini-Mab purification using IMAC. For PC purification study, Korah used pure PC instead of Cohn Fraction IV-1. PC purification yield using Mab (70 %) was 7 times better than that using mini-Mab's (10 %).

For the overall economic analysis, Korah (2003) estimated that PC purification using the mini-Mab is 40 times cheaper than that using regular Mab.

Table 14. An economic analysis of the production for the mini-Mab using *E. coli* and for the Mab using animal cell culture. (modified from Korah's analysis, 2003)

Mini-Mab		Mab	
Total cost of Media (\$/L)	6.09	Total cost of Media (\$/L)	60.18
2XTY	3.45	DMEM*	27.5
IPTG	1.82	Calf serum	30.0
Ampicillin	0.59	Antibiotic	2.68
Glucose	0.23		
Production time (day)	1	Production time (day)	4
Production level (g/L)	0.45	Production level (g/L)	0.1
Molecular weight (kDa)	30	Molecular weight (kDa)	150
Overall production rate (mole/day/dollar)	$2.5 \times 10^{-6}$	Overall production rate (mole/day/dollar)	$2.8 \times 10^{-9}$

\*Dulbecco's modification of Eagle's Media

Since Korah's preliminary study, there has been more optimization in the mini-Mab production and the new production rate of the mini-Mab was estimated to be 1000 times better than that of Mab's (Table 14). The mini-Mab purification yield from the source of the reactor broth was improved and became 34 % by commercially available protein A affinity chromatography. The yield of PC purification from Cohn Fraction IV-1 is 16 %.

With these new improvements, PC purification using mini-Mab was re-estimated as approximately 100 times cheaper than that using regular Mab (Table 15).

Table 15. Economic analysis of PC purification using mini-Mab and regular Mab.

	Mini-Mab	Mab
Production cost	1	1000
Antibody purification	2	1
PC purification	4.5	1
Overall ratio	9	1000

## CHAPTER V

### CONCLUSIONS

The following Conclusions were made for PC production process from Cohn Fraction IV-1 using mini-Mab produced by recombinant *E. coli* in this study.

Mini-Mab production. Out of the thirteen HB2151 *E. coli* colonies, E4FX was confirmed to be the colony producing the mini-Mab the most and was selected for the optimization of the mini-Mab production.

The optimum conditions for the mini-Mab production were determined to be 0.1 % glucose; 0.1 mM IPTG; initial pH 5.5 ~ 6.0; the harvest time of the 18 hrs; and media temperature at 23 ~ 30 °C. The mini-Mab production level was increased from 15 mg/L to 450 mg/L after the optimization of the culture conditions. PMSF in the production media negatively affects the mini-Mab production. The best way of storing the mini-Mab produced media is at -70 °C. If the media has to be stored at 4 °C for some reason, the cells should be removed first and stored with PMSF.

Mini-Mab Purification. Using commercially available protein A Sepharose, the mini-Mab was purified from the supernatant of the production media at a yield of 34 %.

PC purification. PC purification performance was tested for the mini-Mab using four commercially available gel matrices. For pure PC, NHS Sepharose<sup>TM</sup> showed the best PC purification efficiency of 54 %.

The optimum amount of the mini-Mab for PC purification was determined to be 4 mg of mini-Mab per 1 ml of NHS Sepharose<sup>TM</sup> gel matrix.

Cohn Fraction IV-1 is an inexpensive source for PC. The amounts of PC in the fraction were determined to be 104 mg-PC/kg-paste. When Cohn Fraction IV-1 is used, PC purification yield using the mini-Mab immobilized NHS Sepharose<sup>TM</sup> was 16 %, much lower the case with pure PC. When samples of the PC and PC homologues mixture with and without HSA were tested, the yield of 25 % was obtained without HSA, while 13 % was obtained with HSA. DEAE anion exchange chromatography was able to remove 99.8 % HSA from Cohn Fraction IV-1 and 65 % of PC was retained in the eluate. Also PC was concentrated approximately 10 times during the process. PC purification by the mini-Mab immobilized NHS Sepharose<sup>TM</sup> showed 25 % of the PC purification yield from the eluate of DEAE chromatography of Cohn Fraction IV-1.

A brief economic analysis showed that the PC purification cost using the mini-Mab might be approximately 100 times cheaper than that by regular Mabs.

## **CHAPTER VI**

### **FUTURE WORK**

The following studies are suggested for future studies, to improve PC purification using mini-Mab.

Mini-Mab production. It is suggested to produce mini-Mab in a single step instead of the two stages of growth and production. Scale-up of mini-Mab production may be beneficial, especially using bioreactors.

Mini-Mab purification. Mini-Mab purification using IMAC may also be studied as an inexpensive process.

PC purification. Recycling of the PC source material back to the column during the adsorption step may minimize the loss of PC in the source material. Mechanism of HSA interfering PC purification may be investigated. Developing mini-Mab immobilization methods with less affinity reduction may increase the purification yield significantly.

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## **NOMENCLATURE**

aPC: Activated Protein C

BCA: Bicinchoninic Acid

BSA: Bovine Serum Albumin

CNBr: Cyanogen bromide

DEAE: Diethylaminoethyl

DI water: Deionized Water

DMEM: Dulbecco's Modification of Eagle's Medium

E. coli: Escherichia coli

ELISA: Enzyme Linked Immunosorbent Assay

FII: human blood factor II

FVII: human blood factor VII

FIX: human blood factor IX

FX: human blood factor X

FDA: Food and Drug Administration

Fv: Variable Fragment

HIT: heparin induced thrombocytopenia

HRP: Horseradish Peroxidase

HSA: Human Serum Albumin

ICUs: Intensive Care Units

IDA: Iminodiacetic Acid

IgG: Immunoglobulin G

IMAC: Immobilized Metal Affinity Chromatography

IPTG: Isopropyl  $\beta$ -D-thiogalactopyranoside

LPS: Lipopolysaccharides

Mab: Monoclonal Antibody

Mini-Mab: Mini-Antibody

MW: Molecular Weight

NHS: Normal Hydroxysuccinimide

NTA: Nitrilotriacetic acid

O.D.: Optical Density

OPD: O-phenylenediamine Dichloride

PC: Protein C

PCC: Prothrombin complex concentrate

PEG: Polyethylene Glycol

pI: Isoelectric point

PMSF: Phenylmethylsulfonyl Fluoride

rhPC: Recombinant Human PC

scFv: Single-chain Variable Fragment

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TBS: Tris Buffered Saline

TBST: Tris Buffered Saline with Tween 20

TEMED: Tetramethylethylenediamine

$V_H$ : Variable Region in a Heavy Chain

$V_L$ : Variable Region in a Light chain

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## PRESENTATIONS:

Kang, K.A., Korah, L.K., and **Ahn, D.G.** (2001). Optimization of the Culture Conditions and Purification of anti-Protein C-miniantibodies produced from *E. coli*. *International Society of Oxygen Transport to Tissue (ISOTT)*, Aug. 11~15, Philadelphia, PA.

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